HARVESTING TIME INFLUENCES HEALTH-PROMOTING PROPERTIES, AND NANOEMULSIONS INHIBIT *E. COLI* O157:H7 GROWTH ON SPINACH

A Thesis

by

NOORANI BARKAT

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Bhimanagouda S. Patil
Co-Chair of Committee,	G. K Jayaprakasha
Committee Member,	Matthew Taylor
Head of Department,	Dan Lineberger

May 2017

Major Subject: Horticulture

ABSTRACT

Spinach (*Spinacia oleracea*) is a leafy vegetable, rich in health-promoting compounds such as flavonoids, carotenoids, chlorophylls, ascorbic acid, vitamins, and dietary fiber. In the first study, we measured levels of various phytochemicals in spinach at different growing times. Spinach was harvested at 20, 30, 40, 50, and 60 days after planting. Our results demonstrated that nitrate levels increased from 20 (1909 μg g^{-1}) to day 40 (3668 μg g^{-1}) then decreased (974 μg g^{-1}) at day 60. Lutein, chlorophyll *a* and *b* were higher in 60 day samples and β-carotene contents were higher at 50 days. Twelve flavonoids were identified. Total phenolics ranged from 885 to 1162 μg/g. Spinach at 30, 40, and 60 days inhibited α-amylase activity at rates similar to acarbose (inhibitor). Bile acid binding potential was maximal in all samples. This study showed that harvest time has major effect on phytochemical levels and mature spinach has highest phytochemical levels.

The second study aimed to prepare stable nanoemulsions (NE) using grapefruit and lemon essential oils (EO) to evaluate their antimicrobial properties against *Escherichia coli* O157:H7. Encapsulation of these EO's in NE aided inhibition of pathogen growth on spinach during refrigerated (5 °C) storage. Particle sizes of grapefruit and lemon NE were 303 and 155 nm, respectively. Spinach samples were inoculated with *E. coli* O157:H7, and then treated with one of three different treatments (control, 200 ppm chlorine, 5% grapefruit oil NE or 3% lemon oil NE [v/v]). The efficacy of the antimicrobial treatments was determined according to *E. coli* O157:H7 survival at 0, 3, 6, and 9 days of storage. Chlorine and 3% lemon NE exhibited greater

inhibitory effects against *E. coli* O157:H7 versus the grapefruit NE. Nonetheless, all treatments produced significant reductions in pathogen numbers on spinach compared to untreated controls. Significant reduction was observed on each storage day with chlorine treatment and 3% lemon NE with 2.4 and 1.6 $\log_{10}$-cycles reduction on day 0, respectively, and the highest reductions were observed on 9^{th} day of storage with 4.3 and 5.4 $\log_{10}$-cycles reduction. These findings indicated the potential of these natural EO for decontamination of fresh produce.

DEDICATION

I would like to dedicate this thesis to my parents for empowering me and for giving me wings to explore to come this far from Pakistan, my brothers and sisters for their support throughout this period of time.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Bhimanagouda S. Patil, co-chair Dr. G.K Jayaprakasha and member of my graduate committee, Dr. Matthew Taylor for their continuous support and for helping me in understanding research in a broader way. Their encouragement motivated me to do a better job than expected.

I want to thank all my colleagues, especially Dr. Singh, Priyanka and Rita who were always there to assist me with research questions and their help is something I will always remember and will try my best to help others the way they helped me.

My special thanks to my great friends Ali, Ankita and Shaista who were always there on my side to support me morally and for being there with me when I needed my family around me. They motivated me when I thought research is not easy and they kept on telling me that you can do it.

I would like to mention my mentor from University of Agriculture Faisalabad, Dr. Amjad, who assisted me during my undergraduate studies and encouraged me to apply abroad for masters to explore in a better way.

Finally, my deepest respect and thanks to my father and mother who believed all their children, especially daughters, that gender is just a word and to my siblings who supported me in both my ups and downs.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by my graduate committee: Dr. Bhimanagouda S. Patil (chair), Dr. G.K Jayaprakasha of the Department of Horticultural Sciences and Dr. Matthew Taylor from the Department of Animal Science at Texas A&M University, College Station, Texas, USA. All other work conducted for thesis was accomplished by the student under the supervision of advisors.

Funding Sources

This work was made possible by the United States Agency for International Development (USAID) through UC-Davis under grant number 201400223 and USDA NIFA under Grant Number 2015-67013-22813.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES vi

LIST OF FIGURES ix

LIST OF TABLES xi

CHAPTER I INTRODUCTION 1

Objectives 2

CHAPTER II LITERATURE REVIEW 3

CHAPTER III HARVESTING TIME AFFECTS THE PHYTOCHEMICAL LEVELS, FREE RADICAL SCAVENGING ACTIVITY, AMYLASE INHIBITION, AND BILE ACID BINDING CAPACITY OF SPINACH (SPINACIA OLERACEA) 4

Introduction 4
Materials and Methods 7
Plant material 7
Chemicals 7
Quantification of ascorbic acid (AA) and dehydro ascorbic acid (DHA) 8
Analysis of nitrate 9
Quantification of carotenoids 9
Determination of flavonoids 10
DPPH free radical scavenging activity 10
ABTS assay 11
Determination of total phenolics 11
Inhibition of α-amylase 12
Bile acid binding ability of spinach 12
Fiber analysis 13
LC-ESI-HR-QTOF-MS analysis 14

Statistical analysis 15
Results and Discussion 15
Ascorbic acid and DHA 15
Nitrate 16
Carotenoids 18
Flavonoids 19
Radical scavenging activities 21
Total phenolics 22
α-Amylase inhibition 23
Bile acid binding 24
Identification of flavonoids 27

CHAPTER IV GRAPEFRUIT AND LEMON OIL NANOEMULSIONS INHIBIT ESCHERICHIA COLI O157:H7 GROWTH ON SPINACH DURING REFRIGERATED STORAGE 32

Introduction 32
Materials and Methods 34
Materials 34
Optimization and formulation of emulsions 34
Particle size, polydispersity index (PDI), and pH 35
Loading Capacity of Essential Oil in Nanoemulsion 35
Sample preparation 35
Preparation of media for pathogen survival analysis 37
E. coli O157:H7 inoculum preparation 38
Inoculation of spinach 38
Statistical analysis 39
Results 39
Optimization of nanoemulsions and their characterization 39
Loading capacity 42
Antimicrobial activity of treatments 46
Conclusion 48

CHAPTER V CONCLUSION 49

REFERENCES 51

LIST OF FIGURES

Page

Figure 1. Ascorbic acid and total ascorbic acid levels in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± S.E of three samples. Means with the same letter do not significantly differ ($P < 0.05$) 16

Figure 2. Levels of nitrate in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± standard error of three samples. Means with the same letter indicate no significant differences between treatments ($P < 0.05$) 17

Figure 3. Chlorophyll b, a, lutein and beta carotene in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment ($P < 0.05$) 18

Figure 4. Free radical scavenging activity from methanol (flavonoid) and acetone (carotenoids) extracts from samples harvested on different days. The results (Least square means ± SD) are expressed as ascorbic acid equivalents µg/g of samples 22

Figure 5. Phenolic levels in spinach sample on different harvest days. Data are expressed in means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment ($P < 0.05$) 23

Figure 6. Inhibition (%) of α-Amylase inhibition is given in the figure for spinach sample of different harvest days. Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatments ($P < 0.05$) 24

Figure 7. Bile acid binding capacity of 6 different compounds is given in the figure above. Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment ($P < 0.05$) 26

Figure 8. Tandem mass spectra of spinach flavonoids obtained from LC-HR-ESI-QTOF-MS in positive ion mode 30

Figure 9. Proposed fragmentation pattern of identified flavonoids in spinach by LC-HR-ESI-QTOF-MS 31

Figure 10 . Nanoemulsions and their particle size prepared using different concentrations of surfactants with grapefruit essential oil, except the last one, which used 3% lemon oil 40

Figure 11. Survival of E. coli O157:H7 on different storage days following exposure to antimicrobial treatments. Means depict least square means of triplicate replicates with three samples (N=9) and error bars depict one standard deviation from the mean. Means not sharing common letters (a,b,c) differ significantly at $p<0.05$ 48

LIST OF TABLES

Page

Table 1. Levels of flavonoids (1–12) (μg/g) quantified by HPLC from spinach samples harvested on different days 20

Table 2. Composition of emulsions with different surfactants using grapefruit essential oil. * 3% lemon essential oil is used 41

Table 3. Chemical composition of grapefruit nanoemulsion identified and quantified by HS-GC-MS using SPME 44

Table 4. Chemical composition of lemon nanoemulsion identified and quantified by GC-MS using SPME 45

CHAPTER I

INTRODUCTION

Spinach (*Spinacia oleracea* L.) belongs to family *Amaranthaceae*. It is a leafy vegetable and an annual crop that grows up to 30 cm tall. Spinach leaves vary in shape (alternate, simple, ovate, triangular and either flat or curled) and spinach requires cool weather for good growth. It is native to central and southwestern Asia. Both baby spinach and mature spinach are consumed raw as well as cooked in different parts of the world due to its health benefits. Spinach is sold bunched, packaged in bags, canned, or in frozen form. Spinach is highly nutritious and rich in health-promoting compounds such as carotenoids, chlorophylls, nitrate, iron, vitamins, flavonoids, and fiber. [1, 2]

Natural essential oils are often volatile and contain certain characteristic odor compounds formed by secondary metabolites from different parts of plants. Previous studies have shown that essential oils have antimicrobial activities.[3-8] The potential of use of natural essential oils as natural antibacterial agents for food preservation against pathogenic and food spoilage bacteria has recently received attention.[8, 9] Essential oils penetrate into the cell membrane of pathogens and kill them.[10, 11] Essential oils are generally recognized as safe (GRAS) considering they have little to no toxicological effects in food.[12] Citrus essential oil has very potent antibacterial activity[13] due to the presence of citral (3,7-dimethyl-2-7-octadienal), an acyclic α,β-unsaturated monoterpene with two isomers, geranial and neral, both characterized by broad-spectrum antimicrobial activity.[14, 15]

One way to enhance essential oils' biological activity is to prepare nanoemulsions, which combine an oil phase dispersed into an aqueous phase by adding surfactants which help in stabilizing the emulsion.[16] Both grapefruit and lemon nanoemulsions were used to protect fresh produce from different pathogens and control foodborne illnesses.

OBJECTIVES

1. To evaluate the effect of different harvesting times on health-promoting compounds, antioxidant potential, and bile acid binding capacity of spinach.
2. To test citrus nanoemulsions to ensure microbiological safety of spinach during storage against *E. coli* O157:H7 growth.

CHAPTER II

LITERATURE REVIEW

Leafy vegetables, including spinach, are an important part of the human diet due to the presence of health-promoting compounds.[1, 17, 18] Consumption of leafy vegetables has increased, which may potentially produce higher demand globally and demand for year-round availability.[19] People consume baby spinach and mature spinach depending on the geographical location. Based on previous studies, levels of phytochemicals in spinach vary due to different factors such as genotype, variety, temperature, fertilizer, and maturity.[20, 21] Consumers buy both baby spinach (early harvest) and mature spinach (late harvest), but it is important to understand levels of phytochemicals based on maturity of spinach to maximize health benefits.

While enhancing health benefits, it is also important to protect spinach from pathogens such as *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. These pathogens cause foodborne illnesses each year affecting many individuals.[22, 23] Therefore, it is important to determine relevant environmentally friendly decontamination methods. Chlorine is used commercially to decontaminate leafy vegetables but its utility is limited by decomposition produces bearing toxicity concerns and reduction in antimicrobial activity due to interaction with organic matter.[24, 25] To overcome these outbreaks, industries need an effective and environmentally friendly decontaminating method to wash leafy vegetables.

CHAPTER III

HARVESTING TIME AFFECTS THE PHYTOCHEMICAL LEVELS, FREE RADICAL SCAVENGING ACTIVITY, AMYLASE INHIBITION, AND BILE ACID BINDING CAPACITY OF SPINACH (*SPINACIA OLERACEA*)

INTRODUCTION

In humans, a diet containing large quantities of fruits and vegetable is positively correlated with fewer occurrences of chronic diseases, such as cardiovascular diseases and cancer.[18, 26, 27] In addition, fruits and vegetable are good sources of bioactive phytochemicals, essential for maintaining good health. Spinach (*Spinacia oleracea* L.) is a leafy vegetable, traditionally considered to have excellent nutritional qualities due to the presence of carotenoids, chlorophylls, flavonoids, minerals, nitrates, fibers, and vitamins (C, E, and K).[1, 2] In 2015, around 41,890 acres of spinach were cultivated in the U.S, with California producing the most spinach, followed by Arizona, New Jersey, and Texas.[28]

The levels of phytochemicals in fruits and vegetables are affected by environmental factors, genotype, growing conditions, growth stage, and postharvest handling as well as storage conditions.[20, 21, 29, 30] These factors play a vital role in directing total flavonoid content and overall phytochemical levels of the plant. Furthermore, climatic conditions such as average temperature and light strongly affect the chemical composition of horticultural crops.[31]

Studies have observed variation in phytochemical levels in spinach harvested at different growing stages. Changes have been found in ascorbic acid content during plant growth in spinach, but with no consistent pattern.[32] Similarly, higher vitamin C levels were reported in broccoli, which was attributed to higher temperature and radiation conditions.[21] Another study found lower antioxidant activity, due to higher metabolic rates, in the younger leaves of leafy vegetables.[33]

Environmental conditions can have a strong effect on antioxidants. For example, increases or decreases in air temperature before harvesting of spinach leaves can have a significant impact on ascorbic acid (vitamin C) contents,[34] as can other environmental factors that increase reactive oxygen species (ROS).[35] Also, lower light intensity during plant growth leads to low levels of ascorbic acid in tissues.[36] Less-frequent irrigation even leads to higher levels of ascorbic acid in some crops.[37]

In addition to ascorbic acid, other antioxidants also respond to environmental conditions. Similar results were noticed in endive and lettuce with four-fold increases in carotenoid contents in mature leaves; by contrast, in spinach harvested in New Zealand, carotenoid levels were slightly higher in younger leaves than in mature leaves, the variation in carotenoid contents might be due to endive and lettuce being headed with dark green leaves on the outside and light green, younger leaves on the inside, in contrast to spinach, not headed and has leaves growing along the stem with younger leaves being slightly greener than older leaves.[38]

Accumulating evidence also suggests that the composition of flavonoids varies during plant growth.[39] The phenolic and oxygen radical absorbance capacity (ORAC) of genetics, maturity, and harvest conditions.[40]

The addition of nitrogen fertilizers also affects certain phytochemicals in plants. Concentration of nitrate in vegetables depends on fertilizer practice, species, and growing conditions.[41] Changes in nitrate levels have been observed in different seasons, with plants grown in the winter having low nitrate levels as compared to the other seasons.[42] In kale, higher levels of lutein, β-carotene and chlorophyll resulted from the application of nitrogen fertilizer.[43]

During spinach's growing session, the levels of fiber vary and this has a significant impact on the bile acids binding ability. Bile acids solubilize lipids and play an important role in fat absorption, but abnormally high levels make bile acid cytotoxic for human health.[44] Bile acids play a significant role in human health, decreasing plasma cholesterol and improving blood sugar levels in type-II diabetes patients.[45] Bile acids are primarily synthesized in the liver from cholesterol. About 95% of bile acids are reabsorbed[46] and unabsorbed bile acids are excreted from body in the feces.[47] This loss is compensated for by *de novo* bile acid synthesis in the liver.[48] Fiber in the human diet may help in binding bile acids; [49, 50] therefore, we wanted to understand the bile acid-binding ability of spinach harvested on different days. The two type of dietary fiber, soluble and insoluble, have different capacities for binding bile acids. Water-soluble fiber increases excretion of bile acids and water-insoluble fiber decreases excretion of

bile acids.[51] This regulation by binding excess bile acids plays a significant role in human health.

Different phytochemicals also vary over the course of development. The present study reports the comparison of spinach samples harvested on different days, and measures the levels of various phytochemicals, fiber, radical-scavenging capacity, total phenolics, amylase inhibition, and bile acid binding capacity. In addition, 12 flavonoids were characterized by high-resolution mass spectrometry for the first time in spinach at different times during the growing season.

MATERIALS AND METHODS

Plant material

The Samish variety of commercial spinach was cultivated at J&D Produce (Edinburgh, TX) and harvested at regular intervals of 20, 30, 40, 50, and 60 days after planting, and shipped to the Vegetable and Fruit Improvement Center (VFIC), Texas A&M University, College Station, TX overnight in refrigerated conditions. Spinach samples were washed thoroughly in water to remove the soil, stem part removed and only leaf was crushed in the pestle and mortar using liquid nitrogen and stored at -20 °C until used for experimentation.

Chemicals

Methanol, acetone, L-ascorbic acid, chlorophyll a, chlorophyll b, lutein, β-carotene, sodium nitrate, quercetin 3, 4, di-O-glucoside, meta phosphoric acid (MPA),

pepsin, α amylase, pancreatin, sodium cholate (CA), sodium deoxycholate (DCA), sodium glycochenodeoxycholate (GCDCA), sodium chenodeoxycholate (CDCA), sodium glyco deoxycholate (GCDA), sodium glycocholate (GCA), sodium chloride, ammonium nitrate, potassium dihydrogen phosphate, potassium chloride, potassium citrate, uric acid sodium salt, urea, lactic acid sodium salt and porcine gastric mucin, were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals used were of analytical grade.

Quantification of ascorbic acid (AA) and dehydro ascorbic acid (DHA)

Crushed samples (4 g) were mixed with 6 mL of 3% MPA and vortexed for a min, sonicated for 60 min and then centrifuged for 15 min at 8000 RCF (Beckman Model TJ-6).[52] The supernatant was passed through a 0.45 µm filter prior to HPLC analysis. Dehydroascorbic acid (DHA) was analyzed by mixing 300 µL of the extract with 300 µL of tris (2-carboxyethyl) phosphine (TCEP). AA and DHA were analyzed by Thermo HPLC with a Photo Diode Array (PDA) detector (Finnigan surveyor), auto sampler plus (Finnigan surveyor), and MS pump, using an isocratic method. Separation of AA and DHA was achieved using an Eclipse column (XDB-C18, 4.6×150 mm, 5-µm particle size) using 0.03 M phosphoric acid as mobile phase at a flow rate of 400 µL/min. The ascorbic acid peak was monitored at 244 nm and levels were quantified by regression equation and dilution factor.

Analysis of nitrate

Samples for analysis of nitrate content were prepared by extracting a known amount of spinach (2 g) with 2 mL of nanopure water by sonication for 1 h, followed by centrifugation. The supernatant was separated and passed through 0.45-µm filter. Extract (5 µL) was injected into an Eclipse column (XDB-C18, 4.6×150 mm, 5 µm particle size), and analysis was performed by Thermo HPLC. Elution was carried using 0.03 M phosphoric acid as the mobile phase. The flow rate was 400 µL/minute and the nitrate peak was monitored at 254 nm. Quantification of nitrate was done using sodium nitrate as the standard.

Quantification of carotenoids

Extraction of carotenoids (lutein, chlorophyll a, chlorophyll b, and β-carotene) was carried out using acetone as per the method described by Kidmose et al.[53] Each spinach sample (5 g) was extracted with 10 mL of acetone by vortexing for 30 s and sonicated for 30 min in dark conditions, then centrifugation was carried out at 800 g for 15 min. After, decanting and filtering, the residue was re-extracted with 5 mL of acetone. Both extracts were combined, filtered through a 0.45-µm PTFA filter, and transferred to an amber vial for HPLC analysis.

A Waters HPLC was used, equipped with a binary pump, detector, and auto-sampler. Carotenoids were separated using a binary gradient mobile phase (A) MeOH and (B) tert-butyl methyl ether. A C30 column (150×4.6 mm, 3-µm particle size) was

used. The injection volume was 30 µL with a flow rate of 0.5 mL/min. Carotenoids are light sensitive, so the extraction was carried out in dark conditions.

Determination of flavonoids

Ten g of crushed sample was extracted with 20 mL of methanol and the mixture was vortexed for 1 min, sonicated for an hour, and finally centrifuged for 15 minutes (Beckman Model TJ-6, Ramsey, MN, USA). The residue was re-extracted with 10 mL of methanol. Both extracts were passed through 0.45 µm PTFA syringe filters. Flavonoid analysis was performed with a Waters HPLC (Milford, MA, U.S.A.) connected to a PDA detector with 717 plus auto sampler and binary HPLC pump. Flavonoids were separated in symmetry column (C-18, 3.9×150 mm, 5-µm particle size), 20 µL was injected into the HPLC with a flow rate of 0.4 mL/min. Elution was performed using 0.03 mM phosphoric acid (A) and 1:1 of acetonitrile: water (B) as a mobile phase and chromatogram was recorded at 340 nm. The quantification of each flavonoid was expressed relative to querecetin-3-O-glucoside.

DPPH free radical scavenging activity

Both flavonoids (methanol) and carotenoids (acetone) extracts were used for measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity.[54] Ten microliters of different concentrations of ascorbic acid (equivalent to 5, 10, 20, 40, 80, 100 µg) and 10 µL of spinach extract (methanol and acetone) were pipetted into different wells of a microplate and the total volume was adjusted to 100 µL

by adding methanol. Then, 180 µL of DPPH was added and absorbance recorded at 515 nm using a microplate reader. Results were expressed in mg of ascorbic acid (AA) equivalents per gram of sample.

ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reagent was prepared by mixing 7 mmol/L aqueous ABTS solution and 2.45 mmol/l potassium persulfate solution.[54] The mixed solution was stored in the dark for 16 h. Fresh ascorbic acid standard (0.05 mg/mL) was prepared in 3% of MPA. Methanolic and acetone extracts were used to evaluate ABTS radical scavenging activities according to our previous publication.[54]

Determination of total phenolics

The total phenolic acid content of spinach samples was determined using Folin–Ciocalteu reagent (FCR).[54] Gallic acid (0, 10, 20, 30, 40, 50, 75, and 100 µg) was used as a standard for preparation of a calibration curve for total phenolic content and nano pure water was used to adjust the total volume to 100 µL. A sample of 10 µL extract was added and the volume was adjusted to 100 µL by adding MeOH. Afterward, 40 µL of FCR was added to all wells and incubated for 10 min and finally, 50 µL of sodium carbonate was added further incubated for 20 min. Plates were read in a microplate reader at 760 nm.

Inhibition of α-amylase

Inhibition of amylase was measured according to Maeda et al[55] with some modifications. Dextrose (10 µL) was used to prepare a standard curve at different concentrations (equivalent to 25, 50, 75, 100, 125, and 150 µg) and the total volume of each well was adjusted to 230 µL. For analysis of samples, 20 µL of methanolic extract was pipetted into each well in triplicate, saline was added to adjust volume to 140 µL, 45 µL of starch (10 mg/mL) followed by 45 µL of amylase (10 mg/mL) was added to initiate the reaction. The plate was incubated for an hour at 25 °C. The color was developed by adding 50 µL of DNSA to all samples and standard wells. The color was developed by heating the plate at 75 °C for 1 h. Blank wells were prepared without starch to subtract the any reaction between samples and assay mixture. After developing the color, the plate was read at 540 nm. A positive control using acarbose and a negative control using MeOH was prepared for the calculation of inhibition percentage.

Bile acid binding ability of spinach

Known amounts (2 g) of freeze dried spinach samples were treated with 2 mL water and 10 mL simulated saliva fluid in buffer (pH 6.8) according to published protocols.[56] After mixing for 10 min at 37 °C in a horizontal shaker, the sample was acidified by 1M HCl to pH 2, to mimic the stomach digestion, followed by addition of pepsin. Then the samples were vortexed and kept on a shaking water bath for 1 h. For the intestinal phase, pH was adjusted to 6.9 with NaOH, 5 mL of pancreatin (6.25 mg/mL in 50 mM phosphate buffer) and 4 mL of a bile acid mixture[56] containing CA,

DCA, GCDCA, CDCA, GCDA and GCA were added and incubated for 3 h at 37 °C on a shaker at 200 rpm. Later, enzyme was inactivated at 80 °C for 7 min and centrifuged at 10,000 RCF for 15 min, decanted the liquid and reside was washed with excess water to remove adhering bile acids.

The residue contains the bound bile acids, which were released by extracting with 80% aqueous methanol by sonication followed by centrifugation. This step was repeated once more and both extracts were pooled, concentrated under vacuum to get 4 mL and passed through a 0.45-µm filter and injected into the HPLC. Bound bile acids were quantified by HPLC (Agilent 1200 series, Foster City, CA, USA) using Gemini C-18, 5 µm column (250 mm x 4.6 mm i.d) with a guard column, (Phenomenex, Torrance, CA, USA). The separation was performed using a binary mobile phase (A) 0.03 M phosphoric acid and (B) acetonitrile, with a flow rate of 0.7 mL/min. For the samples, 20 µL was injected and peaks were monitored at 210 nm.

Fiber analysis

Fiber content was analyzed by Medallion labs (Golden Valley, MN, USA) according to the A2LA accredited ISO/IEC 17025 protocol.[57] In brief, duplicate samples were digested with three enzymes (α-amylase, protease, and amyloglucosidase) to remove starch and protein. Solid particles not in solution after digestion are considered insoluble dietary fiber and are measured by gravimetric analysis. The filtrate from the above step was collected and precipitated with ethanol to obtain soluble dietary fiber.

The soluble fraction was then filtered and measured gravimetrically. Total dietary fiber was calculated as the sum of the insoluble and soluble percentages.

LC-ESI-HR-QTOF-MS analysis

The methanol extracts were analyzed for flavonoids with a 1290 Agilent LC (Agilent, Santa Clara, CA) equipped with PDA detector and coupled with a maXis impact high-resolution mass spectrometer (Bruker Daltonics, Billerica, MA). The chromatographic separations were carried on a Zorbax eclipse plus C_{18} column (3 µm, 100 × 2.0 mm) at 65°C with a flow rate of 100 µL/min. The mobile phase was composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in water: acetonitrile (1:1). The solvent gradient was: 0 to 2% B (10 min), 2% to 45% B (12 min), 45% to 60% B (5 min), 60% to 90% B (4 min), 90% to 0% B (3 min) and 100% isocratic A (5 min). Mass spectral analyses were performed using electrospray ionization (ESI) in positive ion mode. A mass range from *m/z* 50–2000 amu was applied to obtain the mass spectra. The ion source capillary voltage was set at 4.5 kV, nebulizer gas pressure was 2.1 bar and drying gas flow rate 8.0 L/min. Nitrogen was used for both nebulizer and drying gas. The MS and bbCID collision energy were set at 49-140 eV. External instrument calibration was performed with a sodium formate solution. Nine sodium formate clusters were used in the calibration using a high-precision calibration mode and 1 µL sample was injected.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS software. Significant difference was tested and means were compared using Tukey's Honestly Significant Differences (HSD) test at 5% probability levels (0.05) using SPSS software. The results are expressed as mean ± SEM.

RESULTS AND DISCUSSION

Ascorbic acid and DHA

The levels of ascorbic acid and total ascorbic acid content in spinach samples harvested at different time points were analyzed by HPLC (Figure 1A, 1B). The ascorbic acid levels and contents varied significantly ($P < 0.05$) during the maturation and growing periods. Low levels of ascorbic acid were found in 20-, 30- and 40-day samples, but ascorbic acid increased significantly at 50 (50.55 µg/g) and 60 days (64.57 µg/g). A similar trend was observed for total ascorbic acid, with an increase at later harvest days or with maturity. Maturity at harvest seems to be a significant factor in ascorbic acid levels.[58] A previous study found that levels of ascorbic acid were lower in summer as compared to winter for early-spring spinach[59], even though levels of ascorbic acid positively correlate with light intensity in fruits and vegetables. In another study, a decrease in the air temperature from 5 to -5 °C for ten days before harvesting spinach led to an increase in levels of vitamin C from 50 to 175 mg per 100 g FW.[34] Water stress before harvesting can, however, reduce total ascorbic acid levels.[60] These study results

indicate that harvest weather conditions have a great impact on the levels and total amounts of ascorbic acid.

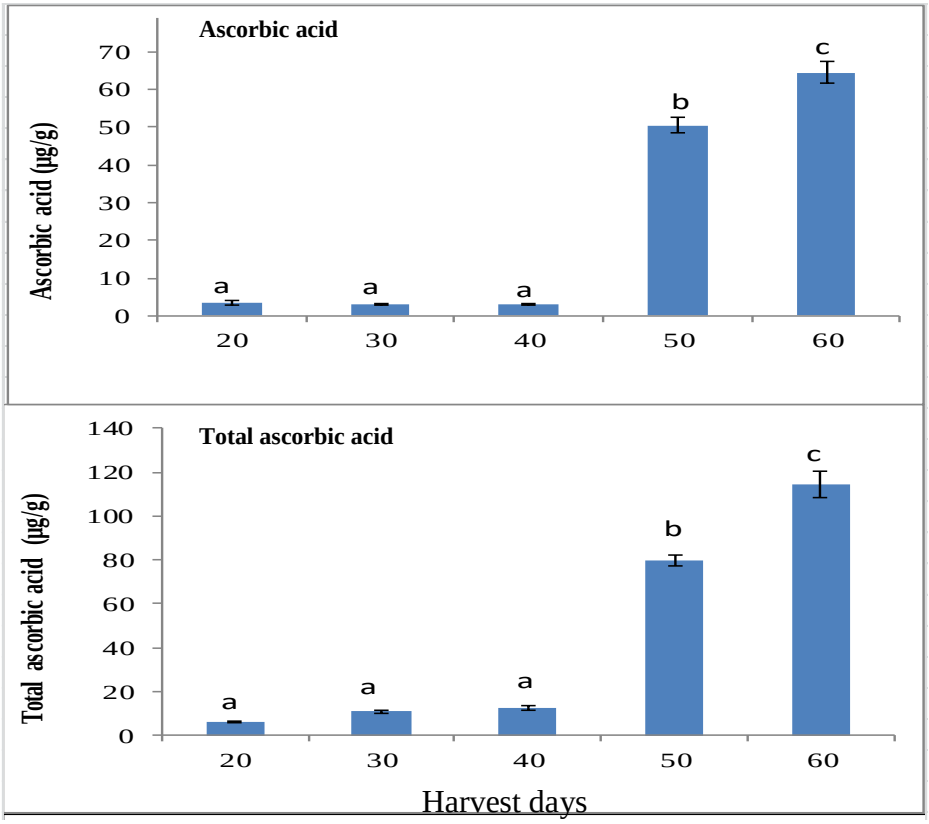

Figure 1. Ascorbic acid and total ascorbic acid levels in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± S.E of three samples. Means with the same letter do not significantly differ *(P < 0.05)*

Nitrate

The nitrate levels were measured in spinach samples harvested on different days. Nitrate levels increased from day 20 (1909 µg/g) to day 40 (3668 µg/g) and then decreased at day 60 (974 µg/g). The 40-day samples had the highest nitrate level and the 60-day samples had the lowest levels (Figure 2). Results indicated the lowest nitrate

contents in late-harvested spinach. A similar pattern was found in a study by Amr et al. [61], who showed late-harvested spinach cultivars had the lowest nitrate contents, but the trend was not consistent. Another study showed that summer-grown spinach had higher levels of nitrate compared with winter to early spring spinach.[59] The levels of nitrates were different in organically grown versus conventionally grown salad and rocket leaves. Agronomic practices and environmental factors also affect nitrate contents in spinach, including time of application of nitrogen fertilizer, herbicides, temperature, water, season, and location.[62]

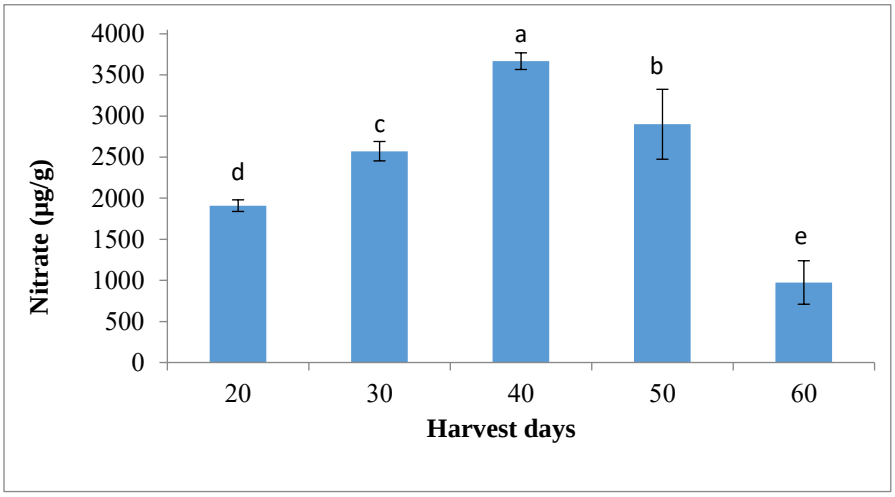

Figure 2. Levels of nitrate in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± standard error of three samples. Means with the same letter indicate no significant differences between treatments *(P < 0.05)*

Carotenoids

In leafy vegetables, the predominant carotenoids are lutein, β-carotene, violaxanthin, and neoxanthin as well as chlorophyll a and b. The levels of chlorophyll b, lutein, and chlorophyll a were highest in the 60-day samples and β-carotene levels were highest in the 50-day samples. Figure 3 shows the levels of carotenoids and chlorophylls quantified in the spinach samples harvested on different days. In general, ripening and maturation of fruits and vegetables leads to an increase in biosynthesis of carotenoids (carotenogenesis). [63]

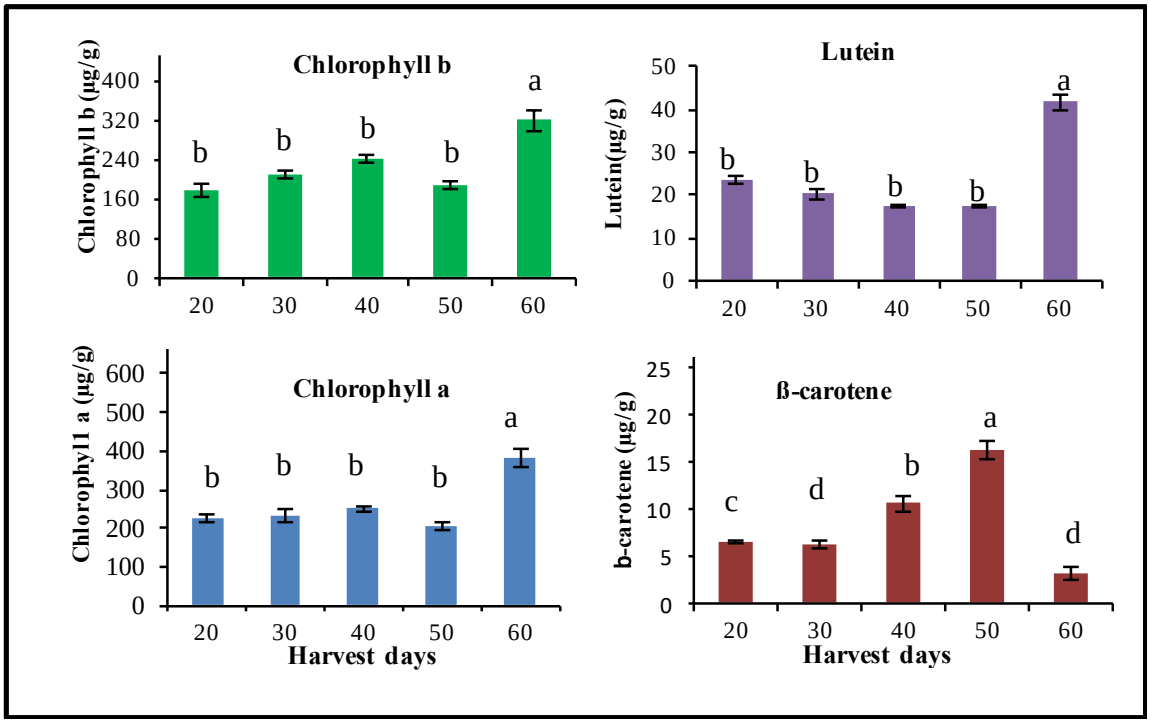

Figure 3. Chlorophyll b, a, lutein and beta carotene in spinach sample on different harvest days (20, 30, 40, 50 and 60). Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment *($P < 0.05$)*

Changes were detected in carotenoid levels, including lutein and β-carotene, and chlorophylls a and b, amongst samples harvested on different days. Chlorophyll a and chlorophyll b showed the same trend: levels increased from day 20 to day 40, then decreased on day 50 and significantly increased on day 60. By contrast, lutein levels decreased starting from day 20 until day 50 of harvest, and finally increased on day 60. β-carotene levels showed an opposite trend, increasing from day 20 to 50 and decreasing at day 60. Azevedo et al.[38] found higher carotenoids content in young leaves of New Zealand spinach versus in mature leaves. Conditions under which crop is grown also effects phytochemical levels, hydroponic lettuce grown under polythene covering to lower the temperature had decreased carotenogenesis, resulting in low levels of lutein and β-carotene.[64]

Flavonoids

For the quantification of flavonoids, samples were extracted with methanol and injected into the HPLC. Twelve different flavonoids were identified and by LC-MS and their respective peaks were quantified. The total flavonoid content in spinach was 588–954 µg/g during the harvest period. The flavonoid 5, 3, 4-trihydroxy-3, methoxy-6, 7-methylendioxyflavone-4-ß-D-glucuronide was prominent (23-36%) of the total flavonoid content, followed by patuletin-3-O-β-D-glucopyranosyl-(1→6)-[β-D-apiofuranosyl-(1→2)]-β -D-glucopyranoside (10-17%) during the spinach harvesting period. In the present study, trends of most of the flavonoids varied: from day 20 to 30, flavonoid contents increased, and then decreased at day 40 harvest, but increased again

at day 60. The reason might be that flavonoids accumulate in younger leaves and then decrease due to the dilution that occurs with leaf growth.[39]

Table 1. Levels of flavonoids (1–12) (μg/g) quantified by HPLC from spinach samples harvested on different days

Day	20	30	40	50	60
1	61.33±1.3^{a}	135.48±2.7	109.58±4.0^{c}	75.85±3.8^{b}	150.94±3.7^{e}
2	19.61±0.2^{a}	70.1±3.1b^{c}	63.7±3.8b^{c}	33.85±1.2^{b}	104.7±4.0^{d}
3	12.94±1.0^{a}	35.93±3.1^{c}	30.78±1.6^{c}	25.76±3.3^{b}	51.78±1.8^{d}
4	9.22±0.3^{d}	11.2±0.4^{e}	4.15±0.6^{b}	2.46±0.1^{a}	7.87±0.2^{c}
5	23.6±1.3^{d}	68.28±2^{e}	47.3±2.4^{b}	29.29±0.8^{a}	70.5±1.4^{c}
6	47.13±0.9^{a}	66.59±2.1^{b}	66.27±5.6^{b}	50.77±1.4^{a}	94.12±1.7^{c}
7	36.1±2.5^{b}	38.7±1.3^{c}	23.9±1.9^{a}	24.7±0.7^{a}	40.9±1.1^{c}
8	80.79±1.8^{d}	78.45±2.6^{c}	49.9±2.9^{a}	68.51±1.5^{b}	79.48±1.1^{d}
9	10.6±0.8^{d}	9.17±0.7^{c}	5.43±0.5^{a}	6.96±0.3^{b}	10.3±0.3^{d}
10	210.8±10.2^{c}	213.9±14.3^{a}	169.94±7.6^{b}	168.25±4.6^{a}	219.83±3.9^{d}
11	8.82±0.5^{b}	10.34±0.6^{bc}	10.65±0.8^{bc}	7.5±0.4^{a}	8.72±0.3^{a}
12	47.72±3.4^{ab}	46.23±3.4^{a}	55.27±4.1^{bc}	44.09±2.3^{a}	55.47±5.4^{c}
Total	588.78	814.58	677.06	588.06	954.85

Twelve flavonoids quantified using HPLC as follows,

1) patuletin-3-O-β-D-glucopyranosyl-(1c 6)-[β-dapiofuranosyl-(1 → 2)]-β-D-glucopyranoside
2) patuletin-3-O-β-D-glucopyranosyl-(1c 6)-β-D-glucopyranoside
3) spinacetin-3-O-β-D-glucopyranosyl-(1→6)-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside

4) patuletin-3-O-β-D-(2″-β-coumaroylglucopyranosyl-(1→6)-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside

5) spinacetin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside

6) patuletin-3-O-ranosidecopyranosyl-(11copyranosyl6)-β-D-glucopyranoside

7) spinacetin-3-O-β-D-(2″-feruloylglucopyranosyl-(1→6)-β-D-glucopyranoside

8) spinatoside

9) jaceidin-4-β-D-glucuronide

10) 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4′-β-D-glucuronide

11) 5,4′,4 - lucuronideay-3, methoxy-6:7-methylendioxyflavone-4--glucuronide

12) 5, 4′,4 - Dihydroxy-3-methoxy-6:7-methylendioxyflavone-4-ß-D-glucuronideide

Radical scavenging activities

Free radical scavenging potentials of methanol and acetone extracts of spinach samples were analyzed (Figure 4). The DPPH and ABTS radical scavenging activities are expressed as ascorbic acid equivalents. The DPPH and ABTS radical scavenging activities were higher at 30 and 60 days for both the methanolic and acetone extracts, compared to the samples harvested on the other days. The trend for DPPH radical scavenging activity was almost same for both methanolic and acetone extracts, with a minor increase in levels on day 30 in methanol-extracted samples. Flavonoids and phenolics from spinach react with nitrogen-centered free DPPH radical with a characteristic absorption at 517 nm, and convert it to 1, 1,-diphenyl-2-picryl hydrazine, due to its hydrogen-donating ability at a very rapid rate. The degree of discoloration

indicates the scavenging potential of the antioxidants. These free radical scavenging activities of the spinach samples are attributed to their hydrogen-donating ability.[65]

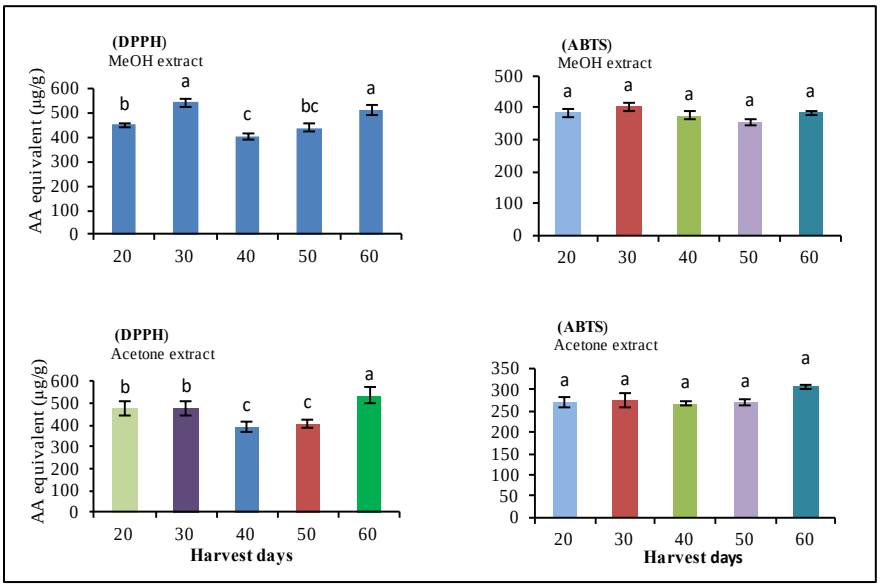

Figure 4. Free radical scavenging activity from methanol (flavonoid) and acetone (carotenoids) extracts from samples harvested on different days. The results (Least square means ± SD) are expressed as ascorbic acid equivalents µg/g of samples

Total phenolics

Total phenolic contents of the spinach samples ranged between 885 and 1162 µg/g. The pattern of total phenolic content was inconsistent: the levels of total phenolics increased from day 20 to day 40 and then decreased on day 50 but increased again on day 60. Spinach harvested on day 40 had the highest total phenolics and the spinach harvested on day 20 had the lowest (Figure 5). These results confirmed that mature spinach has higher phenolic contents than baby spinach. A study done by Howard *et al*, spinach planted in late fall and harvested in spring showed more total phenolics and

antioxidant capacity compared to spinach planted in early fall and harvested in late fall; these differences in levels are likely due to growing conditions, as well as biotic and abiotic stresses.[40]

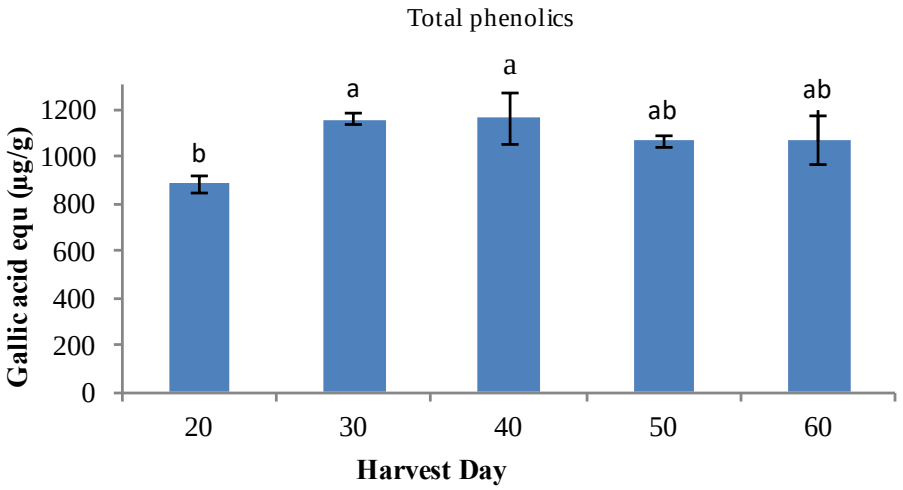

Figure 5. Phenolic levels in spinach sample on different harvest days. Data are expressed in means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment *(P < 0.05)*

α-Amylase inhibition

The enzyme α-amylase can affect people with type-2 diabetes.[66] Therefore, the α-amylase inhibitory action of spinach was harvested on different days was also evaluated. The α-amylase inhibitory activity was determined by a 96-well plate method and the results were compared with a standard inhibitor, acarbose. The results demonstrate that inhibition of α-amylase varied significantly on each harvest day with an inconsistent pattern (Figure 6).

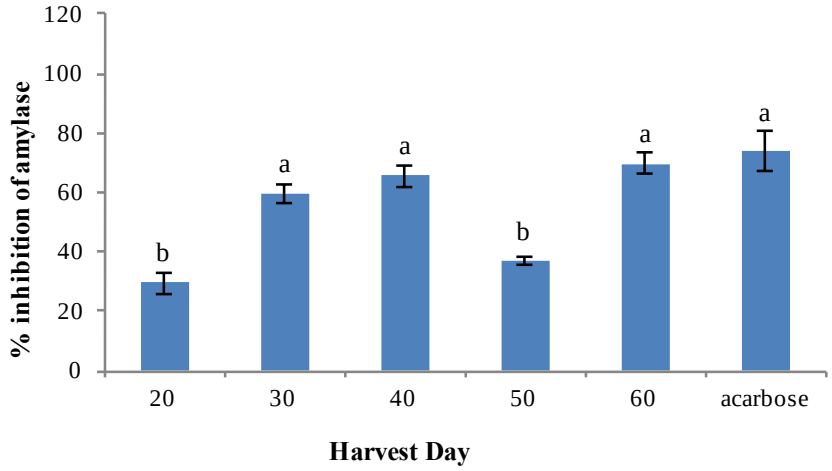

Figure 6. Inhibition (%) of α-Amylase inhibition is given in the figure for spinach sample of different harvest days. Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatments *(P < 0.05)*

The highest α-amylase inhibition was observed at day 60 (85%) and the lowest was observed at day 20. The percent inhibition increased from day 20 to 40; then, after decreasing on day 50, an increase was observed on day 60. These findings indicate that spinach harvested mid-season has more α-amylase inhibitory activity. Inhibition of α-amylase activity inhibits the conversion of carbohydrates into simple sugars, which can help diabetic patients who have abnormally high levels of sugar in their blood,[67] as controlling α-amylase activity can help to maintain their sugar levels.

Bile acid binding

The percentage of bound bile acids varied in the samples. The samples harvested at 30 and 50 days bound significantly higher amounts of GCDCA and GDCA, compared

with the other samples (Figure 7). The amount of GCA bound was higher at 20 and 40 days and decreased in 30-, 50-, and 60-day samples. The amount of GCA bound decreased from day 20 to day 30, increased at day 40, and decreased again day 60. The amounts of CA, GCDCA, and GCDA bound by the spinach samples showed the same trend: their levels increased from day 20 to 30, decreased at day 40, and then increased again at day 60. The amount of CDCA that was bound increased till day 40 and decreased on day 50, with a slight increase on day 60; the highest amount of this bile acid was bound by samples harvested on day 30 and bound DCA levels were highest for samples from day 30 and 40. The bound bile acids varied significantly at different harvest days for all the compounds tested.

The levels of soluble and insoluble fibers levels likely are responsible for the binding ability. In the spinach samples, soluble and insoluble fiber increased with maturity with a higher percentage of insoluble fiber, compared to soluble fiber, at all harvest days. Levels of soluble and insoluble fiber at day 30 were 10.1% and 20.5%, respectively, the lowest levels of all harvest days. On day 40, the fiber content (soluble fiber: 10.9% and insoluble: 21.1%) was slightly higher compared to the previous time point. The same trend was observed on day 50, but the difference in level was greater than that observed the previous days with 12.8% soluble fiber and 24.3% insoluble fiber. The fiber content at day 60 was 13.9% soluble fiber and 23.7% insoluble fiber, the highest levels observed.

Kahlon et al.[68] reported the bile acid binding capacity of green bell pepper, spinach, and various members of the family *Brassicaceae* (cabbage, kale, etc.); the

highest binding was observed in spinach. However, this study did not determine which bile acid was bound to the vegetable sample. Bile acids are cytotoxic when they reach higher levels and, in general, their cytotoxicity is directly proportional to their hydrophobicity. CDCA and DCA are hydrophobic and highly cytotoxic but CA is only cytotoxic at very high levels.[44] Due to their cytotoxic nature, bile acids must be removed from the body when they are produced in high levels. According to our results, the cytotoxic bile acids CDCA and DCA were present in low levels in overall bound bile acid for almost all harvest days with a little bit more on days 30 and 40. CA was also in low levels in bound bile acid compared to rest of bile acids. The variability in levels of different bound bile acids might be due to variation in the profile of phytochemicals in samples from each harvest day.[68] By binding bile acids and stopping them from re-circulating, plant materials can reduce fat absorption, resulting in removal of toxic metabolites and utilization of cholesterol for making more bile acids.[68]

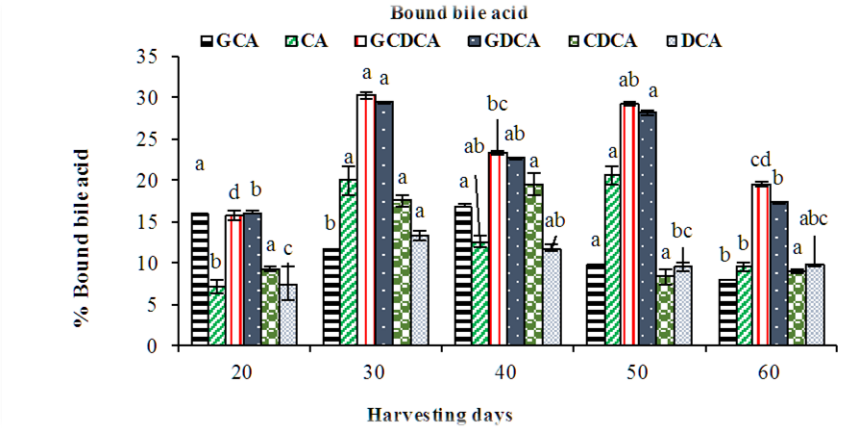

Figure 7. Bile acid binding capacity of 6 different compounds is given in the figure above. Data are expressed in least square means ± S.E of three samples. Means with the same letter indicate no significant differences between treatment *(P < 0.05)*

Identification of flavonoids

In the present research, twelve flavonoids, including patuletin, spinacetin, and glucuronide derivatives, were identified using LC-HR-ESI-QTOF-MS in positive ion mode. Tandem mass spectra of the 12 compounds, along with broad-band collision-induced mass spectra (bbCID) are presented in Figure 8. The possible fragmentation patterns of the 12 compounds are presented in Figure 9. The UV absorption maximum of the identified compounds at 340 nm shows the presence of the flavonoid nucleus. The peak that eluted at RT 10.4 min showed a molecular ion peak at *m/z* 789.2214 $[M+H]^+$ (mass error 16.54 ppm), along with a characteristic aglycone $[A_0]^+$ fragment ion peak at *m/z* 333.0664 $[M+H-456]^+$, which corresponds to the patuletin moiety.[69] On the basis of the mass spectral data and UV spectra, this peak was identified as patuletin-3-O-β-D-glucopyranosyl-(1→6)-β-Dapio-furanosyl-(1→2)]-β-D-glucopyranoside.[70, 71] Similarly, the MS^+ spectrum of the peak that eluted at RT 11.1 min displayed an accurate mass value at *m/z* 657.1774 (mass error 17.19 ppm). The +bbCID spectrum had a characteristic intense base peak at *m/z* 333.0664 $[A_0]^+$. Thus, this peak was identified as patuletin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside. The peaks that eluted at RT 11.7 min and 13.1 min gave mass values at *m/z* 935.2576 (mass error 13.33 ppm) and *m/z* 833.2239 (mass error 12.57 ppm) respectively. The base peak observed in +bbCID spectra at *m/z* 333.0653 (RT 11.7 min) and at *m/z* 333.0646 (RT 13.1 min) corresponds to the patuletin aglycone moiety. It also displays additional, intense fragment ions at *m/z* 147.0464 and at *m/z* 177.0569, which correspond to the loss of the coumaroyl and feruloyl moieties, respectively. Thus, peaks were identified as patuletin-

3-O-β-D-(2"-β-coumaroyl-glucopyranosyl-(1→6)-β-D-apio-furanosyl-(1→2)-β-D-glucopyranoside (RT 11.7 min), and patuletin-3-O-β-D-(2"-feruloyl-glucopyranosyl-(1→6)-β-D-glucopyranoside (RT 13.1 min), respectively.[70-72]

The compounds that eluted at RT 11.2 min and RT 12.2 min exhibited mass values at *m/z* 803.2378 $[M+H]^+$ (mass error -17.19 ppm) and *m/z* 671.1931 (mass error 16.9 ppm) respectively. The mass spectra of both compounds showed a similar fragment ion at *m/z* 347.0819 and at *m/z* 347.08286, respectively which corresponds to the spinacetin moiety.[73] Thus these peaks were identified as spinacetin-3-O-β-D-glucopyranosyl-(1→6)-[β-Dapiofuranosyl-(1 → 2)]-β-D-glucopyranoside and spinacetin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, respectively. Similarly, the peak observed at RT 14.1 min showed a mass signal at *m/z* 847.2411 (mass error 14.18 ppm) and a characteristic spinacetin moiety fragment ion peak at *m/z* 347.0807 $[A_0]^+$. Its spectrum also had a characteristic fragment ion at *m/z* 177.0573, which corresponds to the presence of a ferulic acid residue. Thus, on the basis of the available mass fragmentation and literature reports, this peak was identified as spinacetin-3-O-β-D-(2"-feruloyl-glucopyranosyl)-(1→6)-β-D-glucopyranoside.[70, 74]

The mass spectrum of the peak that eluted at RT 14.3 min exhibits a molecular ion peak at *m/z* 523.1154 $[M+H]^+$ (mass error 13.79 ppm). The base peak signal at *m/z* 347.0821 is potentially formed by the loss of the glucuronide moiety (-176 Da). Thus, on the basis of literature and mass spectrum, this peak was identified as spinatoside.[70, 74] Similarly, the mass spectra of the peaks that eluted at RT 15.0, 16.4, 17.0, and 17.3 min displayed mass signals at *m/z* 537.1311 (mass error 13.43), 521.0943 (mass error 3.39

ppm), 505.0982 (mass error 1.07 ppm), and 535.1114 (mass error 5.92 ppm) respectively. Their +bbCID spectra showed intense base peak signals at *m/z* 361.0963, 345.0614, 329.0661, and 344.0552, respectively, which could be attributed to the loss of the glucuronide moiety from the parent compounds. Thus, the peaks at RT 15.0, 16.4, 17.0, and 17.3 were identified as jaceidin-4'-β-D-glucuronide, 5,3',4' -trihydroxy-3-methoxy-6:7-methylene-dioxyflavone-4'-β-D-glucuronide, 5,4' -dihydroxy-3-methoxy-6:7-methylen-dioxyflavone-4'-β-D-glucuronide, and 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylen-dioxyflavone-4'-β-D-glucuronide, respectively.[70, 74]

In conclusion, our results indicate that the day of harvest has a large impact on the levels of different phytochemicals, fiber content, levels of antioxidants, and bile acid binding capacity. These variations in phytochemicals are due to level of maturity and to factors such as genotype, cultural practices, environmental conditions, irrigation, fertilizer etc. Our results suggest that each phytochemical is higher at different growth stages; this leads to the conclusion that a diet with balanced proportions of young and mature spinach leaves will give maximum health benefits.

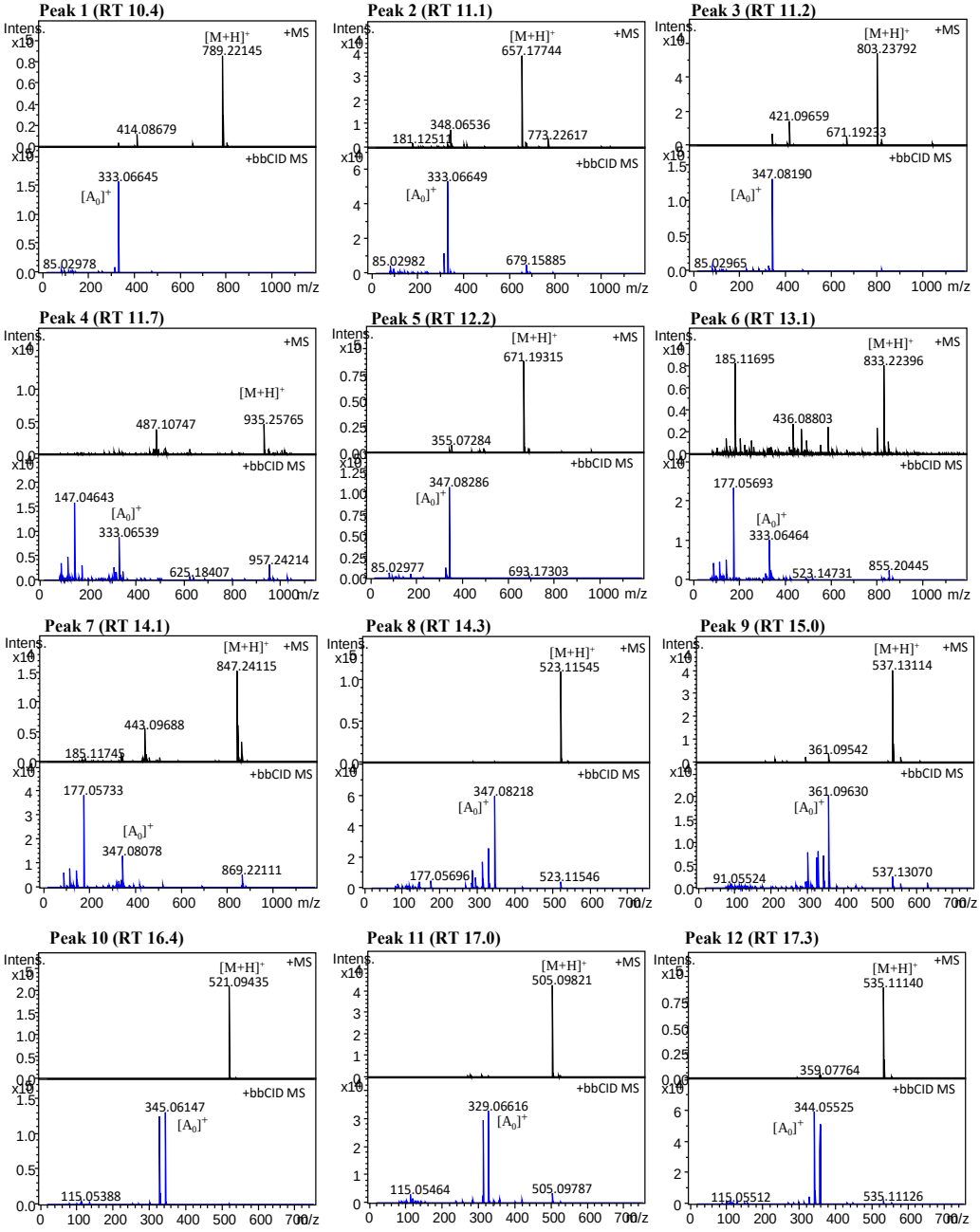

Figure 8. Tandem mass spectra of spinach flavonoids obtained from LC-HR-ESI-QTOF-MS in positive ion mode

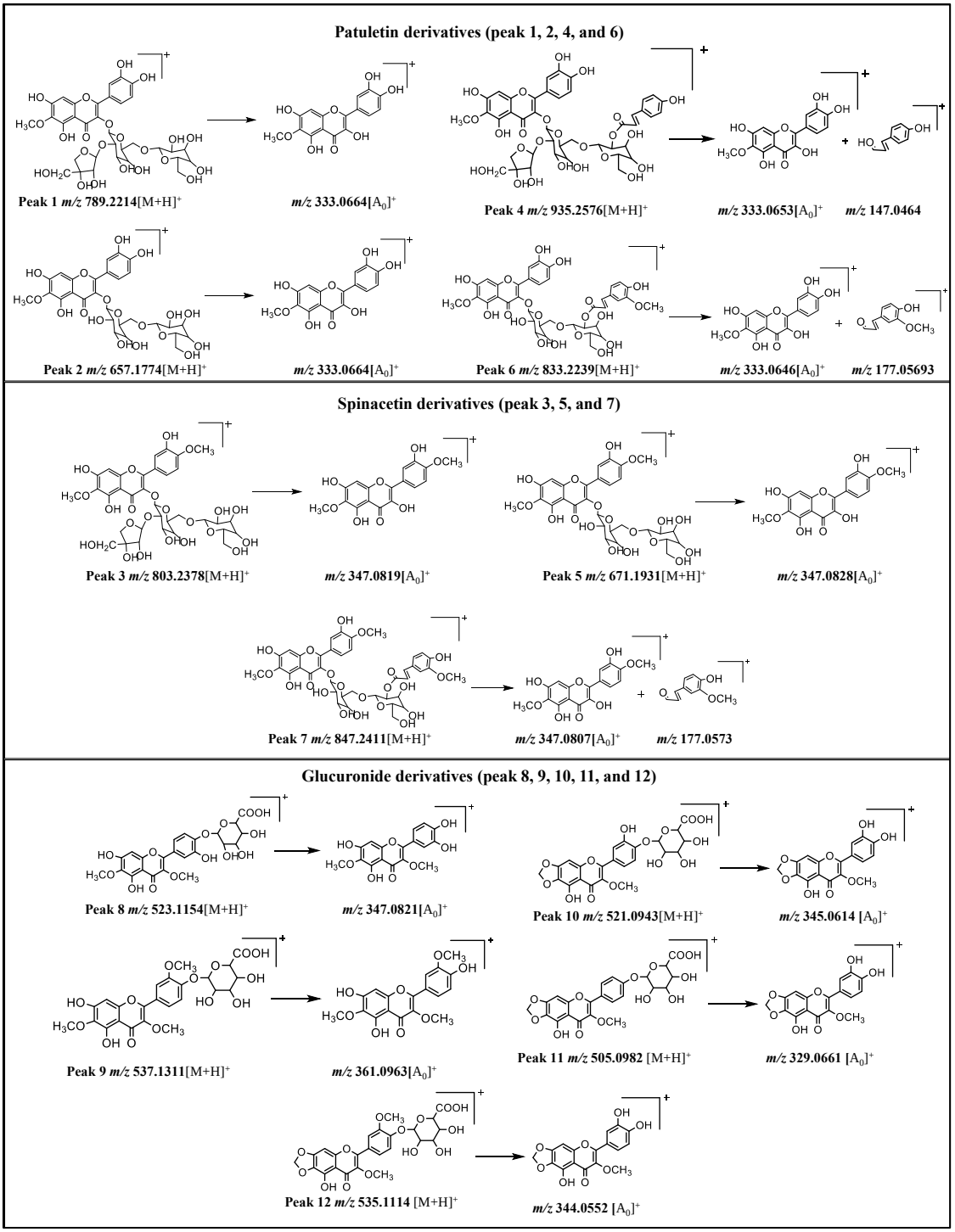

Figure 9. Proposed fragmentation pattern of identified flavonoids in spinach by LC-HR-ESI-QTOF-MS

CHAPTER IV

GRAPEFRUIT AND LEMON OIL NANOEMULSIONS INHIBIT *ESCHERICHIA COLI* O157:H7 GROWTH ON SPINACH DURING REFRIGERATED STORAGE

INTRODUCTION

Fruits and vegetables are an important part of the human diet. Hence, consumption of fresh cut fruits and vegetables has increased worldwide.[75-77]. However, outbreaks of foodborne disease caused by exposure to microbial pathogens such as *Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes* on fresh produce negatively influence human lives each year.[22, 23, 78, 79] In the USA, outbreaks related to fresh produce increased from <1% (1970s) to 6% (1990s). [79-81] In addition, around 30% of the population of industrialized countries gets affected by foodborne illnesses per year and in 2000 around two million people died of diarrheal disease worldwide.[82] These outbreaks have drawn the attention of researchers who aim to develop efficient decontamination methods.

Chlorine is a commonly utilized produce sanitizer, used for washing fresh vegetables and fresh-cut vegetables. However, it creates health concerns as decomposition products may result in formation of carcinogenic halogenated byproducts, and is reported to be inactivated by organic matter.[24, 25] Scientists are looking for environmental-friendly sanitizers that can assure quality and safety of the produce. On the other hand, studies have shown that essential oils and their components from plants, especially aromatic components of plants, have antimicrobial activities.[3-8]

The potential of use of natural essential oils has recently received significant attention for their potential application as natural antibacterial agents for food preservation against pathogenic and spoilage bacteria.[8, 9] Overall, antimicrobial activity of an essential oil is due to the presence of phenolic compounds that interact with the microbial cell membrane.[10] They penetrate into the microbial membrane leading to leakage of ions and cytoplasmic contents, resulting in cell death.[10, 11] Essential oils from many spice-bearing plants are generally recognized as safe (GRAS) since they have little to no toxicological effects in food; they are also biodegradable and biocompatible in nature.[83] Essential oils have received recognition as natural antibacterial agents for food preservation against pathogens and food spoilage bacteria.[8, 9] Citrus essential oil has very potent antibacterial activity[13] due to the presence of citral (3,7-dimethyl-2-7-octadienal), an acyclic α,β-unsaturated monoterpene with two isomers, geranial and neral, and both characterized by a broad spectrum of antimicrobial activity.[14, 15]

To enhance the activity of essential oils against foodborne microbes, they are encapsulated to form nanoemulsions, thereby allowing their contacting of microbes in aqueous phase components of most human foods. The use of nanoemulsions helps in encapsulating, protecting, and improving the activity of essential oil delivery.[84-87] Nanoemulsions consist of two immiscible liquids, oil dispersed in water, with a typical mean droplet size from 100 to 200 nm.[88, 89] Nanoemulsions are stabilized using emulsifiers (surfactants), which function to reduce the surface tension between the phases and allow application of oils into foods. Surfactants can also enhance the solubility of compounds like essentials oil in an aqueous system.[90, 91]

The aim of this study was to formulate stable nanoemulsions of grapefruit and lemon essential oils, and to evaluate their effect against *E. coli* O157:H7. Spinach was used as a model and antibacterial activity was monitored for period of 9 storage days. To the best of our knowledge, this is the first report about evaluation of the antimicrobial activity of grapefruit and lemon nanoemulsions against *E. coli* O157:H7 for 9 days of storage.

MATERIALS AND METHODS

Materials

Lemon and grapefruit cold-pressed essential oils were obtained from Texas Citrus Exchange (Mission, TX, USA). Tween 20 and sorbitan monolaurate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Baby spinach was purchased from a College Station, Texas, grocer. Microbiological media, specifically tryptic soy agar (TSA), tryptic soy broth (TSB) and peptone were purchased from Becton, Dickinson and Co. (Sparks, MD, USA). The antibiotic rifampicin and 100% methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Optimization and formulation of emulsions

Different nanoemulsions were prepared by using various concentrations of essential oil and surfactants. Surfactant emulsions (1 or 5%) were prepared using surfynol 485, cytoguard LA 20, sodium dodecyl sulfate (SDS), or Tween 20 loaded with 1% grapefruit essential oil. The double-surfactant emulsions were prepared for

formulation of 1% and 5% grapefruit oil and 3% lemon emulsion. Similarly, emulsions of 3% lemon and 5% grapefruit essential oil were prepared. First both 0.61% Tween 20 (hydrophilic phase) and 0.51% of sorbitan monolaurate (lipophilic phase) were prepared in nanopure water separately by stirring on a magnetic stirrer for 4 h at 500 rpm. Then, 3 mL of lemon essential oil was added drop-by-drop to 25 mL of the lipophilic phase and stirred for 30–60 min, followed by 75 mL of hydrophilic phase. The mixture was stirring for another 12–14h at 25°C to obtain a stable emulsion. The 5% grapefruit emulsion was prepared the same way except 5 ml of grapefruit oil was used.

Particle size, polydispersity index (PDI), and pH

Particle size and PDI for all nanoemulsions were measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern instruments, Westborough, MA). All emulsions were diluted 20-fold with nanopure water, vortexed for one minute, and transferred to plastic 1 cm path length cuvettes. Particle size was calculated using Zetasizer software (version 7.02). Both particle size and PDI were recorded in triplicate. The pH was also measured for all emulsions.

LOADING CAPACITY OF ESSENTIAL OIL IN NANOEMULSION

Sample preparation

Nanoemulsion samples for GC-MS were prepared in triplicate by taking 25 mg of the respective nanoemulsion, which was mixed with 1 mL of nanopure water and 5

μL of 250 ppm perillyl alcohol then vortexed and transferred into 20 ml vials and analyzed in GC-MS.

Loading capacity and composition of both oils were analyzed by headspace – solid phase micro-extraction (HS-SPME) GC-MS using a Thermo Scientific Triplus autosampler, Trace Ultra GC, and DSQ II mass spectrometer (Thermo Finnigan, Thermo Fisher Scientific, Inc., San Jose, CA, USA). HS-SPME was conducted using 50/30 μm divinylbenzene /carboxen / polydimethylsiloxane (DVB/CAR/PDMS) fiber. The SPME fiber was initially conditioned in the GC injector at 225°C for 1 h according to manufacturer's recommendations. The vial containing sample was preheated at 60 °C while agitating for 30 sec. The SPME fiber was then exposed to the HS of the vial for 5 min at 60 °C for the adsorption of volatiles. The SPME fiber was introduced into the inlet of the GC for 2 min at 225 °C to desorb the volatile compounds in splitless mode. Post-conditioning was done for 12 min after each sample. The injector port temperature was maintained at 225 °C, while the column temperature was ramped twice as follows: 50 °C for 1 min and increased to 170 °C at the rate of 6 °C/min, further raised to 225 °C at the rate of 25°C/min and held for 1 min. Volatile compounds were separated on a fused silica Zebron ZB-WAXPlus capillary column (30 m × 0.25 mm, 0.25 μm film (Phenomenex, CA, USA) coated with bonded 100% polyethylene glycol. Helium was used as a carrier gas at a flow rate of 1 mL/min and run time was 24 min. The ion source temperature was maintained at 285 °C for EI mode. The ionization voltage was 70 eV, the mass range was 45-400 amu and the scan rate was 12.82 scans/sec. The relative area was calculated using Thermo xcalibur software version 2.2 SP1.48. The volatiles were

identified on the basis of their retention indices, which were calculated by injecting hydrocarbons (C8 – C24) under the same program and operating conditions according to the published method[92]. The volatile components were identified by comparing kovats indices (KI), retention times of authentic standards, and matching the spectral fragmentation patterns in Wiley library database and published mass spectra [93-95]. Further, chemical composition and loading capacities of both oils in emulsions were calculated.

Preparation of media for pathogen survival analysis

The virulent pathogen *E. coli* O157:H7 strain, resistant to rifampicin (0.1 g/L) was obtained from the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University, College Station, TX, USA) culture collection and revived from tryptic soy agar (TSA) slant culture by scraping a loop-full of culture into a tube containing 10 mL sterile tryptic soy broth (TSB), followed by incubation for 24 h at 35 °C.

Tryptic soy agar supplemented with 0.1 g/L rifampicin (TSAR) was prepared by first dissolving powdered medium base in distilled water according to the manufacturer's instructions. Dissolved medium was then sterilized at 121 °C for 15 min, after which it was tempered in a flowing water bath to 46 °C. Rifampicin was dissolved at 0.1 g into 5.0 ml MeOH, and the entire volume was added to 1.0 L of sterile, tempered TSA, followed by stirring. Petri dishes were then poured and allowed to cool prior to refrigerated storage (5 °C) or immediate use in microbiological analysis of pathogen

survival from treated spinach. In instances where Petri dishes containing TSAR were refrigerated prior to use, plates were removed prior to initiation of the experiment to allow them to temper to ambient conditions and check for contamination on medium surfaces.

E. coli O157:H7 inoculum preparation

E. coli O157:H7 was revived in tryptic soy broth (TSB) as described above. Following incubation, the entire volume was aseptically transferred to a sterile 15 mL conical tube and centrifuged for 15 min at 3500 RCF at ambient temperature. The resulting supernatant was decanted and 9.5 mL of 0.1% (w/v) peptone water was added to the remaining bacterial pellet, and then vortexed for 1.0 min to suspend the pellet for subsequent inoculation.

Inoculation of spinach

Samples of spinach (25 g each) were transferred into sterile plastic containers. Four different treatments were tested: control (inoculated, untreated), T1 (chlorine, 200 ppm, 25°C), T2 (5% [v/v] grapefruit nanoemulsion) and T3 (3% lemon oil nanoemulsion). Four sets of samples were prepared (one per treatment) with three samples per replicate for each treatment for testing of *E. coli* O157:H7 survival post-treatment after 0, 3, 6, and 9 days of storage at 5 °C. All spinach samples were spot-inoculated with the *E. coli* O157:H7 inoculum for all storage days with 100 µL (10 µL/spot x 10 spots), and were given 2.0 h for pathogen attachment prior to testing or

refrigerated incubation. For all samples, *E. coli* O157:H7 survivors were determined by preparation of serial dilutions in 0.1% peptone diluent and spreading of dilutions on surfaces of TSAR. Inoculated plates were incubated for 24 h at 35 °C and *E. coli* O157:H7 colonies were then counted.

Statistical analysis

Colony counts were first $\log_{10}$-transformed prior to statistical analysis. The experiment was treated as a full factorial, and replicated three times identically (n=3; N=9). Treatments were statistically compared by analysis of variance (ANOVA) and significantly differing means were separated using Tukey's Honestly Significant Differences (HSD) (p<0.05) using SPSS.

RESULTS

Optimization of nanoemulsions and their characterization

Different formulations of nanoemulsions were prepared using different surfactants and different concentrations of essential oil. Each emulsion had different appearance as shown in Figure 10. Grapefruit (1%) emulsion using 1% SDS resulted in thicker emulsion and 5% SDS gave a very thin and transparent emulsion. Using 5% surfynol 485 gave thicker nanoemulsion than 1% surfynol 485; the 1% and 5% cytoguard emulsions were similar in appearance. Both emulsions using cytoguard LA 20 and combination of sorbitan monolaurate and Tween 20 gave similar emulsions that were white and thick. The emulsions using 1% and 5% surfynol 485 both resulted in

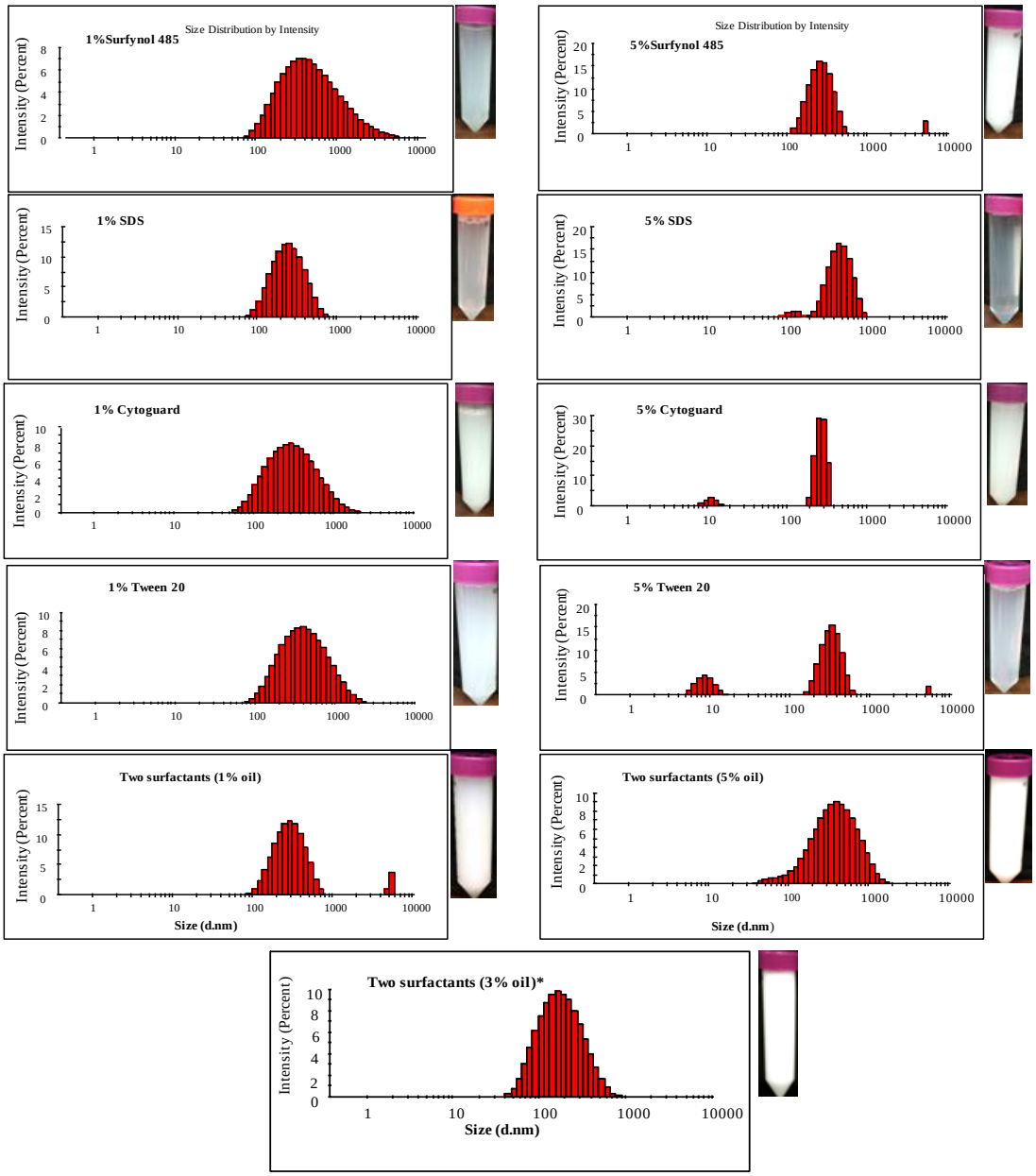

Figure 10. Nanoemulsions and their particle size prepared using different concentrations of surfactants with grapefruit essential oil, except the last one, which used 3% lemon oil

slightly thin white emulsions. Lemon (3%) and grapefruit (5%) emulsions were prepared using Tween 20 and sorbitan monolaurate, which remained stable for a longer period of time; they were then selected further for the spinach storage study. The stability of nanoemulsions mainly depend on the nature of the oil, the surfactant and the concentration of surfactant.[96] The nanoemulsions also showed varying results for each different concentration of surfactant. The type of essential oil also effected nanoemulsion formulation depending on levels of chemical composition of hydrocarbons and oxygenated hydrocarbons.

Table 2. Composition of emulsions with different surfactants using grapefruit essential oil. * 3% lemon essential oil is used

#	Surfactants	Composition (%)	Oil added (%)	Particle size (d.nm)	PDI	pH
1.	Surfynol 485	1	1	216	0.13	4.79
2.	Surfynol 485	5	1	397	0.41	5.26
3.	SDS	1	1	223	0.16	4.68
4.	SDS	5	1	465	0.43	4.56
5.	Cytoguard LA 20	1	1	224	0.44	2.97
6.	Cytoguard LA 20	5	1	786	0.62	2.64
7.	Tween 20	1	1	337	0.57	3.98
8.	Tween 20	5	1	621	0.59	3.53
9.	Sorbitan mono laurate + Tween20	0.61+0.51	1	361	0.36	3.28
10	Sorbitan mono laurate + Tween20	0.61+0.51	5	303	0.31	3.31
11	*Sorbitan mono laurate + Tween20	0.61+0.51	3	155	0.25	4.55

The average particle size and PDI of nanoemulsions were calculated using Zetasizer and results were presented in Table 2. To test the antimicrobial activity, we used 3% lemon and 5% grapefruit nanoemulsions. Average size and PDI for 3% lemon and 5% grapefruit essential oil nanoemulsion were 155.8 nm, 0.25 PDI and 303 nm, 0.30 PDI. The average size reading for 3% lemon nanoemulsion was smaller compared to 5% grapefruit emulsion and lower readings of PDI suggest that particles are uniformly distributed in emulsions. Highest pH (5.26) was found in 5% surfynol emulsion and lowest was observed in cytoguard LA 20 and the 3% lemon and 5% grapefruit emulsions had pH of 4.55 and 3.31 respectively.

In our study, most of the nanoemulsions with higher concentrations of surfactant had larger droplet sizes, but opposite results were observed by Chang et al.[96] Nanoemulsions that were not stable over period degraded due to Ostwald ripening, in which the oil phase had higher solubility in aqueous phase. Additionally, the droplet size increased with the help of smaller droplets.[97, 98]

Loading capacity

Quantification of both emulsions was performed by GC-MS. Grapefruit (5%) and lemon (3%) emulsions were calculated for presence of D-limonene and perillyl alcohol was used as an internal standard. A total 21 different volatile compounds were identified in both emulsions, including D-limonene, decanal, linalool and ortho-cymene as major compound. We recovered 3.31 and 2.6% of D-limonene from both 5% grapefruit and 3% lemon emulsion. The hydrocarbons are present in grapefruit essential

oil (1330 µg/100 mL) and at higher concentrations than in lemon essential oil (609 µg /100 mL) but levels of oxygenated compounds were higher in lemon oil (372 µg /100 mL) compared to grapefruit oil (80 µg /100 mL).

Citrus includes oranges, mandarins, limes, lemons, and grapefruit.[99] Overall, essential oil of citrus has higher levels of volatiles, compared to other fruits. Monoterpene (d-limonene) and citrus essential oil contain 85–99% volatiles and 1–15% non-volatile components. The volatile constituents are a mixture of monoterpenes (limonene), aldehydes (citral), alcohols (linalool), and esters.[100, 101] Grapefruit oil consists of hydrocarbons with limonene as a major compound, [102] which matches our results. The strong antibacterial activity of oils may be due to the presence of linalool, limonene, and sabinene. It has been reported that these volatiles can destroy the membranes of pathogens and block their respiration.[103, 104]

Table 3. Chemical composition of grapefruit nanoemulsion identified and quantified by HS-GC-MS using SPME

RT	Compound[a]	KI	Relative levels µg/100 mL
5.41	Sabinene	1116	6.28±0.64
6.15	Myrcene	1160	51.63±1.6
6.87	D-limonene	1197	1257.75±74.8
8.5	β-ocimene	1289	14.98±0.23
10.2	heptyl acetate	1368	0.68±0.02
10.62	Nonanal	1386	9.79±1.8
12.28	1-octanal	1472	6.96±2.83
12.75	Decanal	1497	29.47±9.22
13.72	Linalool	1544	11.51±1.12
14.84	terpinen-4-ol	1595	7.52±1.43
15.34	Citral	1623	0.71±0.14
17.32	Carvone	1737	5.11±0.61
18.21	Perillaldehyde	1784	2.93±0.35
19.14	trans-carveol	1840	1.04±0.13
19.25	ethyl laurate	1847	1.57±0.06
19.67	Carveol	1874	0.61±0.07
20.31	perillyl Acetate	1913	0.38±0.04
21.6	Thujopsen	1989	0.35±0.05
22.95	Geranyllinallol	2176	0.24±0.03
23.5	Bisabolene	2257	0.54±0.05

[a]Compounds were listed by elution order in the polar ZB wax column

KI: Kovats indices relative to n-alkanes on polar column

Table 4. Chemical composition of lemon nanoemulsion identified and quantified by GC-MS using SPME

RT	Identified compound[a]	KI	µg /100 mL
4.03	α-pinene	1000	0.74±0.1
5.26	β-pinene	1107	43.05±7.1
6.13	Myrcene	1159	10.92±0.2
6.81	d-limonene	1194	547.80±38.2
10.62	Nonanol	1386	17.68±2.3
11.86	limonene oxide	1434	0.91±0.1
12.33	Citronellal	1475	3.5±0.6
12.75	Decanal	1497	5.4±0.8
13.7	Linalool	1543	29.4±1.3
14.16	pinocarvone	1564	16.4±1.5
14.81	terpinen-4-ol	1593	27.23±2.03
14.49	a-bergamotene	1579	38.7±4.3
15.33	Myrtenal	1816	14±1.5
15.87	trans-pinocarvol	1838	13.7±2
16.31	Citral	1856	21.7±0.8
16.65	α-terpineol	1869	19.8±0.9
17.09	Neryl acetate	1886	91.3±2.4
17.15	gernyl acetate	1888	22.1±4.2
17.32	(-)-carvone	1895	23.2±2.7
17.89	(R)-citronellol	1916	1.94±0.2
19.13	trans carveol	2159	9.8±0.8
19.35	Geraniol	2166	2.3±0.6
21.59	caryophyllene oxide	2237	15.76±1.1
22.95	(-)-spathulenol	2477	2.84±0.1

[a]Compounds are listed by elution order in the polar ZB wax column

KI: Kovats indices relative to n-alkanes on polar column

Antimicrobial activity of treatments

Three different treatments were tested on *E. coli* O157:H7-inoculated spinach samples. As shown in Figure 2, control samples (untreated) maintained consistently higher numbers of *E. coli* O157:H7 across all replicates and storage days as compared to other treatments. Chlorine-treated samples and 3% lemon oil nanoemulsion produced the strongest antimicrobial activity over all storage days, while 5% grapefruit nanoemulsion, although not as effective as the other antimicrobial treatments, was able to nonetheless reduce *E. coli* O157:H7 versus the controls (Fig. 2) ($P<0.05$).

Reductions produced in *E. coli* O157:H7 numbers for each treatment were calculated by subtracting the $\log_{10}$ CFU/g from treated samples on a given sampling day from control readings on the same day. The greatest $\log_{10}$ reduction at the beginning of the experiment was observed in chlorine-treated samples (2.4 $\log_{10}$ CFU/g), whereas reductions for nanoemulsion-treated samples were less at day 0. On storage day 3, pathogen reductions in chlorine-treated samples increased to 3.0 $\log_{10}$ CFU/g, whereas 5% grapefruit nanoemulsion-treated samples and 3% lemon nanoemulsion-treated samples gave reductions of 1.1 and 1.6 $\log_{10}$ CFU/g, respectively. *E. coli* O157:H7 survival trend was same as in day 0. On the 6th day of storage, chlorine treatment led to 3.88 log reductions, 5% grapefruit emulsion resulted in 1.97 log reductions and 3% lemon emulsion showed 3.52 log reductions. The survival trend of *E. coli* O157: H7 was similar to previous storage days with chlorine and lemon emulsion-treated samples with least microbial growth and the control sample with highest *E. coli* 0157: H7 levels. On the last day of storage, the highest log reduction was noticed in 3% lemon nanoemulsion

treatment with 5.4, followed by chlorine treatment with 4.33 and 5% treatment with 2.62 log_{10}-cycle reductions. Control samples had the highest survival values for *E. coli* O157: H7 in all storage days. Statistically, T1 (chlorine treatment) and T2 (3% lemon emulsion) were significantly the same and the other treatments were significantly different and a trend was noticed.

Washing methods for market produce need to be effective and environmentally friendly. Also, the excessive use of chlorine for washing produce causes many environmental and health effects.[24] It is possible that the effectiveness of antibacterial potential of grapefruit and lemon emulsion against *E. coli* O157:H7 could be due to the presence of higher levels of D-limonene or other compounds such as citral or linalool etc. These are results are in agreement with previous studies.[105, 106] Antibacterial activity of emulsions may vary over different storage days due to evaporation of volatile compounds in the essential oil and that can impact its interaction level with microbe. [107]

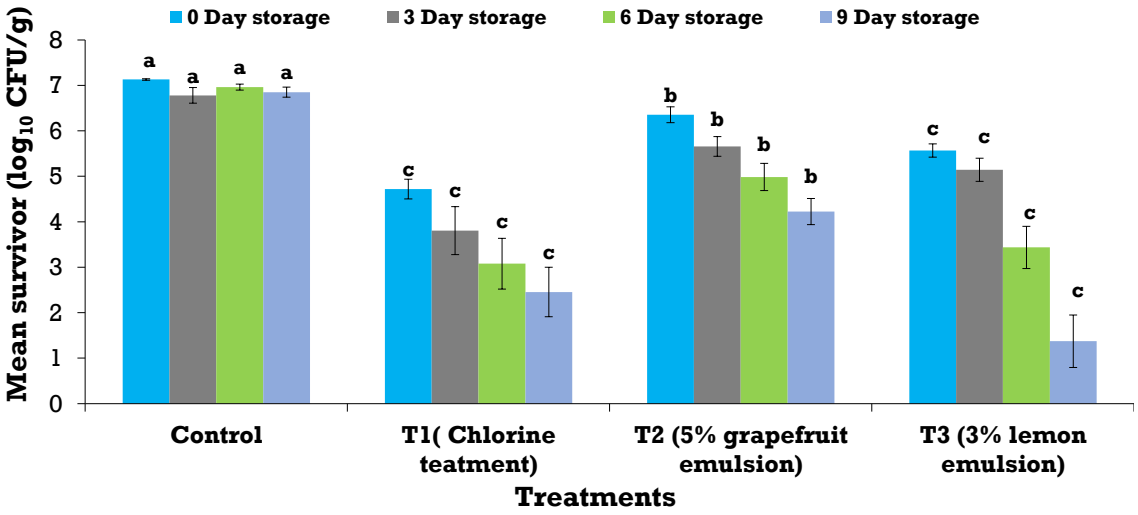

Figure 11. Survival of *E. coli* O157:H7 on different storage days following exposure to antimicrobial treatments. Means depict least square means of triplicate replicates with three samples (N=9) and error bars depict one standard deviation from the mean. Means not sharing common letters (a,b,c) differ significantly at $p<0.05$

CONCLUSION

Overall, different factors contribute to making citrus nanoemulsions effective against *E. coli* O157:H7; these include higher levels of oxygenated hydrocarbons, particle size of nanoemulsions, and stability of emulsions. The stable nanoemulsions were formulated using grapefruit and lemon emulsion and they exhibited very strong inhibitory activity against *E. coli* O157:H7. Much research is still needed in the field of nanoemulsions to make them potentially useful for commercial applications to minimize outbreaks in the future.

CHAPTER V

CONCLUSION

Spinach is an important leafy vegetable, due to its higher demand in market and presence of health-benefiting compounds. Spinach harvested early (baby spinach) and after 25–30 days (mature spinach) was evaluated to measure its health-beneficial properties. The present study demonstrated the significant difference in health-promoting compounds in baby and mature spinach. For example, the levels of vitamin C increased significantly with maturity and nitrate was higher at day 40. Also, chlorophyll a, b and lutein were higher at day 60 and ß-carotene was highest at day 50. This tells us that vitamin c, nitrate, and carotenoids are higher in mature spinach.

Flavonoids and DPPH activity of methanol extract were higher in the 30 and 60 day samples, indicating that consuming either baby or mature spinach can help getting maximum flavonoids and free radical scavenging activity. ABTS did not show significant differences on different harvest days. The phenolic content was higher at 30 and 40 days of harvest while amylase inhibition was higher in 30, 40 and 60 day of harvest, which was equivalent to acarbose. Bile acid binding capacity of six different bile acids compounds changed without any uniform trend on all harvest of days but two bile acid compounds, GCDCA and GDCA, were higher on all harvest days compared to the rest of the bile acid compounds. In conclusion, most of the phytochemicals were higher in mature spinach (late harvest) than baby spinach (early harvest).

After studying impact of different growth stages on phytochemical levels, the second objective was to develop a formulation of green nanoemulsions using citrus essential oil for inhibiting pathogens on leafy vegetables, instead of using chlorine as it is considered to create health concerns. Spinach samples were inoculated with *E. coli* O157:H7, and then treated with one of three different treatments (control, 200 ppm chlorine, 5% grapefruit oil emulsion or 3% lemon oil emulsion [v/v]). Antimicrobial efficacy of treatments was recorded as *E. coli* O157:H7 survival/growth at 0, 3, 6, and 9 days of storage. While chlorine and 3% lemon nanoemulsions exhibited greater inhibitory effects against *E. coli* O157:H7 during storage versus the grapefruit nanoemulsion, they did not differ from one another in pathogen inhibition. Nonetheless, all treatments produced significant reductions in pathogen numbers on each day of refrigerated storage on spinach compared to untreated controls ($P<0.05$). The inhibitory results of both 3% lemon emulsion and 200 ppm chlorine wash were the same. It is possible that produce with 3% lemon nanoemulsion can be protected by this natural nanoemulsion. This may be better strategy for efficient use of waste products of lemon processing, for providing solutions to food safety challenges.

REFERENCES

1. Bottino, A.; Degl'Innocenti, E.; Guidi, L.; Graziani, G.; Fogliano, V., Bioactive compounds during storage of fresh-cut spinach: the role of endogenous ascorbic acid in the improvement of product quality. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 2925-2931.
2. Hord, N. G.; Tang, Y.; Bryan, N. S., Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *The American Journal of Clinical Nutrition* **2009**, *90*, 1-10.
3. Bassolé, I. H. N.; Juliani, H. R., Essential oils in combination and their antimicrobial properties. *Molecules* **2012**, *17*, 3989-4006.
4. Burt, S., Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology* **2004**, *94*, 223-253.
5. Seow, Y. X.; Yeo, C. R.; Chung, H. L.; Yuk, H.-G., Plant essential oils as active antimicrobial agents. *Critical Reviews in Food Science and Nutrition* **2014**, *54*, 625-644.
6. Diao, W.-R.; Hu, Q.-P.; Feng, S.-S.; Li, W.-Q.; Xu, J.-G., Chemical composition and antibacterial activity of the essential oil from green huajiao (Zanthoxylum schinifolium) against selected foodborne pathogens. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 6044-6049.
7. Lv, F.; Liang, H.; Yuan, Q.; Li, C., In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International* **2011**, *44*, 3057-3064.

8. Di Pasqua, R.; Betts, G.; Hoskins, N.; Edwards, M.; Ercolini, D.; Mauriello, G., Membrane toxicity of antimicrobial compounds from essential oils. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 4863-4870.
9. Caillet, S.; Millette, M.; Salmieri, S.; Lacroix, M., Combined effects of antimicrobial coating, modified atmosphere packaging, and gamma irradiation on Listeria innocua present in ready-to-use carrots (Daucus carota). *Journal of Food Protection* **2006**, *69*, 80-85.
10. Salvia-Trujillo, L.; Rojas-Graü, A.; Soliva-Fortuny, R.; Martín-Belloso, O., Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils. *Food Hydrocolloids* **2015**, *43*, 547-556.
11. Juven, B.; Kanner, J.; Schved, F.; Weisslowicz, H., Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Bacteriology* **1994**, *76*, 626-631.
12. Kabara, J., Phenols and chelators. *Food Preservatives, Blackie, Glasgow* **1991**, 200-214.
13. Belletti, N.; Ndagijimana, M.; Sisto, C.; Guerzoni, M. E.; Lanciotti, R.; Gardini, F., Evaluation of the antimicrobial activity of citrus essences on Saccharomyces cerevisiae. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 6932-6938.
14. Hayes, A.; Markovic, B., Toxicity of Australian essential oil Backhousia citriodora (Lemon myrtle). Part 1. Antimicrobial activity and in vitro cytotoxicity. *Food and Chemical Toxicology* **2002**, *40*, 535-543.

15. Wuryatmo, E.; Klieber, A.; Scott, E. S., Inhibition of citrus postharvest pathogens by vapor of citral and related compounds in culture. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 2637-2640.
16. Acosta, E., Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Current Opinion in Colloid & Interface Science* **2009**, *14*, 3-15.
17. Baranowski, T., Understanding the behavioral linkages needed for designing effective interventions to increase fruit and vegetable intake in diverse populations. *Journal of the American Dietetic Association* **2011**, *111*, 1472-1475.
18. Hung, H.-C.; Joshipura, K. J.; Jiang, R.; Hu, F. B.; Hunter, D.; Smith-Warner, S. A.; Colditz, G. A.; Rosner, B.; Spiegelman, D.; Willett, W. C., Fruit and vegetable intake and risk of major chronic disease. *Journal of the National Cancer Institute* **2004**, *96*, 1577-1584.
19. Pollack, S. L., Consumer demand for fruit and vegetables: the US example. *Changing Structure of Global Food Consumption and Trade* **2001**, *6*, 49-54.
20. Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A., Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *Journal of Agricultural and Food Chemistry* **1999**, *47*, 2213-2217.
21. Hakala, M.; Lapveteläinen, A.; Huopalahti, R.; Kallio, H.; Tahvonen, R., Effects of varieties and cultivation conditions on the composition of strawberries. *Journal of Food Composition and Analysis* **2003**, *16*, 67-80.
22. Control, C. f. D.; Prevention, Ongoing multistate outbreak of Escherichia coli serotype O157: H7 infections associated with consumption of fresh spinach--United

States, September 2006. *MMWR. Morbidity and Mortality Weekly Report* **2006**, *55*, 1045.

23. Tauxe, R. V.; Doyle, M. P.; Kuchenmüller, T.; Schlundt, J.; Stein, C., Evolving public health approaches to the global challenge of foodborne infections. *International Journal of Food Microbiology* **2010**, *139*, S16-S28.
24. Gil, M. I.; Selma, M. V.; López-Gálvez, F.; Allende, A., Fresh-cut product sanitation and wash water disinfection: problems and solutions. *International Journal of Food Microbiology* **2009**, *134*, 37-45.
25. Ölmez, H.; Kretzschmar, U., Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *LWT-Food Science and Technology* **2009**, *42*, 686-693.
26. Liu, S.; Lee, I.-M.; Ajani, U.; Cole, S. R.; Buring, J. E.; Manson, J. E., Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The Physicians' Health Study. *International Journal of Epidemiology* **2001**, *30*, 130-135.
27. Voorrips, L.; Goldbohm, R.; van Poppel, G.; Sturmans, F.; Hermus, R.; Van Den Brandt, P., Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study The Netherlands Cohort Study on Diet and Cancer. *American Journal of Epidemiology* **2000**, *152*, 1081-1092.
28. USDA, Vegetable 2016 summary. **2016**.
29. Olsson, M. E.; Ekvall, J.; Gustavsson, K.-E.; Nilsson, J.; Pillai, D.; Sjöholm, I.; Svensson, U.; Åkesson, B.; Nyman, M. G., Antioxidants, low molecular weight carbohydrates, and total antioxidant capacity in strawberries (Fragaria× ananassa):

effects of cultivar, ripening, and storage. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 2490-2498.

30. Patil, B.; Pike, L.; Hamilton, B., Changes in quercetin concentration in onion (Allium cepa L.) owing to location, growth stage and soil type. *New phytologist* **1995**, *130*, 349-355.
31. Klein, B.; Perry, A., Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *Journal of Food Science* **1982**, *47*, 941-945.
32. Bergquist, S. Å.; Gertsson, U. E.; Knuthsen, P.; Olsson, M. E., Flavonoids in baby spinach (Spinacia oleracea L.): changes during plant growth and storage. *Journal of Agricultural and Food Chemistry* **2005**, *53*, 9459-9464.
33. Burton, W. G., *Post-harvest physiology of food crops*. Longman Group Ltd.: 1982.
34. Tamura, A., Effect of air temperature on the content of sugar and vitamin C of spinach and komatsuna. *Horticultural Research (JAPAN)* **2004**, *3*, 187-190.
35. Davey, M. W.; Montagu, M. v.; Inzé, D.; Sanmartin, M.; Kanellis, A.; Smirnoff, N.; Benzie, I. J. J.; Strain, J. J.; Favell, D.; Fletcher, J., Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *Journal of the Science of Food and Agriculture* **2000**, *80*, 825-860.
36. Harris, R. S., Effects of agricultural practices on the composition of foods. *Nutritional Evaluation of Food Processing* **1975**, *2*, 33-57.

37. Stefanelli, D.; Goodwin, I.; Jones, R., Minimal nitrogen and water use in horticulture: Effects on quality and content of selected nutrients. *Food Research International* **2010**, *43*, 1833-1843.

38. de Azevedo-Meleiro, C. H.; Rodriguez-Amaya, D. B., Carotenoids of endive and New Zealand spinach as affected by maturity, season and minimal processing. *Journal of Food Composition and Analysis* **2005**, *18*, 845-855.

39. del Baño, M. J.; Lorente, J.; Castillo, J.; Benavente-García, O.; Marín, M. P.; Del Río, J. A.; Ortuño, A.; Ibarra, I., Flavonoid distribution during the development of leaves, flowers, stems, and roots of Rosmarinus officinalis. Postulation of a biosynthetic pathway. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 4987-4992.

40. Howard, L.; Pandjaitan, N.; Morelock, T.; Gil, M., Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 5891-5896.

41. Greenwood, D. J.; Hunt, J., Effect of nitrogen fertiliser on the nitrate contents of field vegetables grown in Britain. *Journal of the Science of Food and Agriculture* **1986**, *37*, 373-383.

42. Kaminishi, A.; Kita, N., Seasonal change of nitrate and oxalate concentration in relation to the growth rate of spinach cultivars. *HortScience* **2006**, *41*, 1589-1595.

43. Kopsell, D. A.; Kopsell, D. E.; Curran-Celentano, J., Carotenoid pigments in kale are influenced by nitrogen concentration and form. *Journal of the Science of Food and Agriculture* **2007**, *87*, 900-907.

44. Hofmann, A. F., The continuing importance of bile acids in liver and intestinal disease. *Archives of Internal Medicine* **1999**, *159*, 2647-2658.

45. Staels, B.; Kuipers, F., Bile acid sequestrants and the treatment of type 2 diabetes mellitus. *Drugs* **2007**, *67*, 1383-1392.

46. Chiang, J. Y., Bile acids: regulation of synthesis. *Journal of Lipid Research* **2009**, *50*, 1955-1966.

47. Lefebvre, P.; Cariou, B.; Lien, F.; Kuipers, F.; Staels, B., Role of bile acids and bile acid receptors in metabolic regulation. *Physiological Reviews* **2009**, *89*, 147-191.

48. Li, T.; Chiang, J. Y., Bile acids as metabolic regulators. *Current Opinion in Gastroenterology* **2015**, *31*, 159.

49. Eastwood, M.; Hamilton, D., Studies on the adsorption of bile salts to non-absorbed components of diet. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **1968**, *152*, 165-173.

50. Trisat, K.; Wong-on, M.; Lapphanichayakool, P.; Tiyaboonchai, W.; Limpeanchob, N., Vegetable Juices and Fibers Reduce Lipid Digestion or Absorption by Inhibiting Pancreatic Lipase, Cholesterol Solubility and Bile Acid Binding. *International Journal of Vegetable Science* **2016**, 1-10.

51. Ide, T.; Horii, M.; Yamamoto, T.; Kawashima, K., Contrasting effects of water-soluble and water-insoluble dietary fibers on bile acid conjugation and taurine metabolism in the rat. *Lipids* **1990**, *25*, 335-340.

52. Chebrolu, K. K.; Jayaprakasha, G. K.; Yoo, K. S.; Jifon, J. L.; Patil, B. S., An improved sample preparation method for quantification of ascorbic acid and

dehydroascorbic acid by HPLC. *LWT - Food Science and Technology* **2012**, *47*, 443-449.

53. Kidmose, U.; Knuthsen, P.; Edelenbos, M.; Justesen, U.; Hegelund, E., Carotenoids and flavonoids in organically grown spinach (Spinacia oleracea L) genotypes after deep frozen storage. *Journal of the Science of Food and Agriculture* **2001**, *81*, 918-923.
54. Jayaprakasha, G. K.; Girennavar, B.; Patil, B. S., Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. *LWT - Food Science and Technology* **2008**, *41*, 376-384.
55. Maeda, K.; Kakabayashi, S.; Matsubara, H., Complete amino acid sequence of an α-amylase inhibitor in wheat kernel (0.19-inhibitor). *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **1985**, *828*, 213-221.
56. Hur, S. J.; Lim, B. O.; Decker, E. A.; McClements, D. J., In vitro human digestion models for food applications. *Food Chemistry* **2011**, *125*, 1-12.
57. Fiber, Total, Soluble and Insoluble (Gravimetric) analysis, http://www.medallionlabs.com/TestLibrary/Test2.aspx?tid=2326&id=50 **2016**.
58. Jones, R.; Stefanelli, D.; Tomkins, R. In Pre-harvest and post-harvest factors affecting ascorbic acid and carotenoid content in fruits and vegetables, XXIX *International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes* (IHC2014): VI 1106, 2014; 2014; pp 31-42.

59. Fujiwara, T.; Kumakura, H.; Ohta, S.; Yoshida, Y.; Kameno, T., Seasonal variation of L-ascorbic acid and nitrate content of commercially available spinach [Spinacia oleracea]. *Horticultural Research (JAPAN)* **2005**.

60. Mogren, L. M.; Beacham, A. M.; Reade, J. P.; Monaghan, J. M., Moderate water stress prevents the postharvest decline of ascorbic acid in spinach (Spinacia oleracea L.) but not in spinach beet (Beta vulgaris L.). *Journal of the Science of Food and Agriculture* **2015**.

61. Amr, A.; Hadidi, N., Effect of cultivar and harvest date on nitrate (NO 3) and nitrite (NO 2) content of selected vegetables grown under open field and greenhouse conditions in Jordan. *Journal of Food Composition and Analysis* **2001**, *14*, 59-67.

62. Breimer, T., Environmental factors and cultural measures affecting the nitrate content in spinach. *Fertilizer Research* **1982**, *3*, 191-292.

63. Rodriguez-Amaya, D., Nature and distribution of carotenoids in foods. *Developments in Food Science* **1993**.

64. Kimura, M.; Rodriguez-Amaya, D. B., Carotenoid composition of hydroponic leafy vegetables. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 2603-2607.

65. Chidambara Murthy, K. N.; Singh, R. P.; Jayaprakasha, G. K., Antioxidant Activities of Grape (*Vitis vinifera*) Pomace Extracts. *J. Agric. Food Chem.* **2002**, *50*, 5909-5914.

66. Oboh, G.; Ademosun, A., Phenolic extracts from grapefruit peels (Citrus paradisi) inhibit key enzymes linked with type 2 diabetes and hypertension. *Journal of Food Biochemistry* **2011**, *35*, 1703-1709.

67. Odhav, B.; Thangaraj, K.; Khumalo, N.; Baijnath, H., Screening of African traditional vegetables for their alpha-amylase inhibitory effect. *Journal of Medicinal Plants Research* **2013**, *4*, 1502-1507.

68. Kahlon, T.; Chapman, M.; Smith, G., In vitro binding of bile acids by spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage and collards. *Food Chemistry* **2007**, *100*, 1531-1536.

69. Parejo, I.; Jauregui, O.; Viladomat, F.; Bastida, J.; Codina, C., Characterization of acylated flavonoid-O-glycosides and methoxylated flavonoids from Tagetes maxima by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 2801-2810.

70. Cho, M. J.; Howard, L. R.; Prior, R. L.; Morelock, T., Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture* **2008**, *88*, 1099-1106.

71. Watanabe, Y.; Uchiyama, F.; Yoshida, K., Compositional changes in spinach (Spinacia oleracea L.) grown in the summer and in the fall. *Journal of the Japanese Society for Horticultural Science* **1994**.

72. Wang, S.; Liu, L.; Wang, L.; Hu, Y.; Zhang, W.; Liu, R., Structural characterization and identification of major constituents in Jitai tablets by high-performance liquid chromatography/diode-array detection coupled with electrospray ionization tandem mass spectrometry. *Molecules* **2012**, *17*, 10470-10493.

73. Ferreres, F.; Castañer, M.; Tomás-Barberán, F. A., Acylated flavonol glycosides from spinach leaves (Spinacia oleracea). *Phytochemistry* **1997**, *45*, 1701-1705.

74. Koh, E.; Charoenprasert, S.; Mitchell, A. E., Effect of organic and conventional cropping systems on ascorbic acid, vitamin C, flavonoids, nitrate, and oxalate in 27 varieties of spinach (Spinacia oleracea L.). *Journal of Agricultural and Food Chemistry* **2012**, *60*, 3144-3150.

75. Allende, A.; Luo, Y.; McEvoy, J. L.; Artés, F.; Wang, C. Y., Microbial and quality changes in minimally processed baby spinach leaves stored under super atmospheric oxygen and modified atmosphere conditions. *Postharvest Biology and Technology* **2004**, *33*, 51-59.

76. Omac, B.; Moreira, R. G.; Castillo, A.; Castell-Perez, E., Growth of Listeria monocytogenes and Listeria innocua on fresh baby spinach leaves: Effect of storage temperature and natural microflora. *Postharvest Biology and Technology* **2015**, *100*, 41-51.

77. Hanning, I. B.; Nutt, J.; Ricke, S. C., Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathogens and Disease* **2009**, *6*, 635-648.

78. Morrissey, K. M.; Knickrehm, A. K.; Sakata, N. A., Evaluation of listeriosis screening, reporting and education procedures by obstetricians. In 2010.

79. Sivapalasingam, S.; Friedman, C. R.; Cohen, L.; Tauxe, R. V., Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection* **2004**, *67*, 2342-2353.

80. Smith-Palmer, A.; Stewart, J.; Fyfe, L., Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology* **1998**, *26*, 118-122.
81. WHO, N. D., Food safety and foodborne illness. *Biochimica Clinica* **2002**, *26*, 39.
82. Organization, W. H., Food safety and foodborne illness. Fact sheet 237. *World Health Organization, Geneva. Available at: http://www.who.int/mediacentre/factsheets/fs237/en/. Accessed* **2012**, *10*.
83. Sikkema, J.; De Bont, J.; Poolman, B., Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews* **1995**, *59*, 201-222.
84. Donsì, F.; Annunziata, M.; Sessa, M.; Ferrari, G., Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT-Food Science and Technology* **2011**, *44*, 1908-1914.
85. Qian, C.; McClements, D. J., Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. *Food Hydrocolloids* **2011**, *25*, 1000-1008.
86. Rao, J.; McClements, D. J., Food-grade microemulsions, nanoemulsions and emulsions: fabrication from sucrose monopalmitate & lemon oil. *Food Hydrocolloids* **2011**, *25*, 1413-1423.
87. McClements, D. J., Food emulsions: principles, practices, and techniques. *CRC Press:* 2015.

88. Mason, T.; Wilking, J.; Meleson, K.; Chang, C.; Graves, S., Nanoemulsions: formation, structure, and physical properties. *Journal of Physics: Condensed Matter* **2006**, *18*, R635.

89. Shams, N.; Sahari, M. A., Nanoemulsions: Preparation, Structure, Functional Properties and their Antimicrobial Effects. *Applied Food Biotechnology* **2016**, *3*, 138-149.

90. McClements, D. J., Solubilization of oil droplets by micellar surfactant solutions. In *Food Proteins and Lipids*, Springer: 1997; pp 149-159.

91. Torchilin, V. P., Micellar nanocarriers: pharmaceutical perspectives. *Pharmaceutical Research* **2007**, *24*, 1.

92. Adams, R. P., Identification of essential oils by ion trap mass spectroscopy. *Academic Press:* San Diego, 1989.

93. Davies, N. W., Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicon and Carbowax 20M phases. *J. Chromatogr. A* **1990**, *503*, 1-24.

94. Adams, R. P., Identification of essential oil components by gas chromatography/ mass spectrometry. 4 ed.; *Allured Publishing Corporation*: IL, USA, 2007.

95. Jayaprakasha, G. K.; Kumar, C. K.; Chaudhary, P.; Murthy, K. N. C.; Patil, B. S., Identification of volatiles from Kumquats and their biological activities. In *Tropical and Subtropical Fruits: Flavors, color, and Health Benefits*, American Chemical Society: 2013; Vol. 1129, pp 63-92.

96. Chang, Y.; McClements, D. J., Optimization of orange oil nanoemulsion formation by isothermal low-energy methods: influence of the oil phase, surfactant, and temperature. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 2306-2312.

97. Kabalnov, A. S.; Shchukin, E. D., Ostwald ripening theory: applications to fluorocarbon emulsion stability. *Advances in Colloid and Interface Science* **1992**, *38*, 69-97.

98. Julian McClements, D.; Henson, L.; Popplewell, L. M.; Decker, E. A.; Jun Choi, S., Inhibition of Ostwald ripening in model beverage emulsions by addition of poorly water soluble triglyceride oils. *Journal of Food Science* **2012**, *77*, C33-C38.

99. Ahmad, M. M.; Iqbal, Z.; Anjum, F.; Sultan, J., Genetic variability to essential oil composition in four citrus fruit species. *Pakistan Journal of Botany* **2006**, *38*, 319.

100. Smith, D. C.; Forland, S.; Bachanos, E.; Matejka, M.; Barrett, V., Qualitative analysis of citrus fruit extracts by GC/MS: an undergraduate experiment. *The Chemical Educator* **2001**, *6*, 28-31.

101. Flamini, G.; Tebano, M.; Cioni, P. L., Volatiles emission patterns of different plant organs and pollen of Citrus limon. *Analytica Chimica Acta* **2007**, *589*, 120-124.

102. Uysal, B.; Sozmen, F.; Aktas, O.; Oksal, B. S.; Kose, E. O., Essential oil composition and antibacterial activity of the grapefruit (Citrus Paradisi. L) peel essential oils obtained by solvent-free microwave extraction: comparison with hydrodistillation. *International Journal of Food Science & Technology* **2011**, *46*, 1455-1461.

103. Knobloch, K.; Pauli, A.; Iberl, B.; Weigand, H.; Weis, N., Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research* **1989**, *1*, 119-128.

104. Lis-Balchin, M.; Ochocka, R.; Deans, S.; Hart, S., Bioactivity of enantiomers of limonene. *Medical Science Research* **1996**.

105. Zahi, M. R.; El Hattab, M.; Liang, H.; Yuan, Q., Enhancing the antimicrobial activity of d-limonene nanoemulsion with the inclusion of ε-polylysine. *Food Chemistry* **2017**, *221*, 18-23.

106. Donsì, F.; Annunziata, M.; Vincensi, M.; Ferrari, G., Design of nanoemulsion-based delivery systems of natural antimicrobials: effect of the emulsifier. *Journal of Biotechnology* **2012**, *159*, 342-350.

107. Saiyasombati, P.; Kasting, G., Two-stage kinetic analysis of fragrance evaporation and absorption from skin. *International Journal of Cosmetic Science* **2003**, *25*, 235-243.